



## King's Research Portal

DOI:

[10.1136/gutjnl-2019-319850](https://doi.org/10.1136/gutjnl-2019-319850)

*Document Version*

Peer reviewed version

[Link to publication record in King's Research Portal](#)

*Citation for published version (APA):*

Clough, J., Omer, O. S., Tasker, S. D., Lord, G. M., & Irving, P. M. (2020). Regulatory T-cell therapy in Crohn's disease: challenges and advances. *Gut*, 69(5), 942-952. <https://doi.org/10.1136/gutjnl-2019-319850>

### Citing this paper

Please note that where the full-text provided on King's Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher's website for any subsequent corrections.

### General rights

Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the Research Portal

### Take down policy

If you believe that this document breaches copyright please contact [librarypure@kcl.ac.uk](mailto:librarypure@kcl.ac.uk) providing details, and we will remove access to the work immediately and investigate your claim.



## Regulatory T cell therapy in Crohn's disease: challenges and advances

Journal:	<i>Gut</i>
Manuscript ID	gutjnl-2019-319850.R1
Article Type:	Recent advances in basic science
Date Submitted by the Author:	21-Nov-2019
Complete List of Authors:	<p>Clough, Jennie; King's College London, School of Immunology and Microbial Sciences; NIHR Biomedical Research Centre at Guy's and Saint Thomas' NHS Foundation Trust and King's College</p> <p>Omer, Omer; King's College London, School of Immunology and Microbial Sciences; Guy's and Saint Thomas' Hospitals NHS Trust, Gastroenterology</p> <p>Tasker, Scott; King's College London Division of Transplantation Immunology and Mucosal Biology,</p> <p>Lord, Graham; King's College London, School of Immunology and Microbial Sciences; The University of Manchester, Faculty of Biology, Medicine and Health</p> <p>Irving, Peter; Guy's and Saint Thomas' NHS Foundation Trust, Gastroenterology; King's College London, School of Immunology and Microbial Sciences</p>
Keywords:	CROHN'S DISEASE, IMMUNOLOGY, IMMUNOREGULATION, INTESTINAL T CELLS, T LYMPHOCYTES

SCHOLARONE™  
Manuscripts

**Regulatory T cell therapy in Crohn’s disease: challenges and advances**

Jennie N Clough,<sup>\*,1,2</sup> Omer S Omer,<sup>\*,1</sup> Scott Tasker,<sup>1</sup> Graham M Lord,<sup>1,3,#</sup> and Peter M Irving<sup>1,4,#</sup>

- 1. School of Immunology and Microbial Sciences, King’s College London, UK
- 2. NIHR Biomedical Research Centre at Guy’s and St Thomas’ NHS Foundation Trust and King’s College London, UK
- 3. Faculty of Biology Medicine and Health, University of Manchester, UK
- 4. Department of Gastroenterology, Guy’s and St Thomas’ NHS Foundation Trust

**\*Equal contribution**

# Correspondence: [graham.lord@manchester.ac.uk](mailto:graham.lord@manchester.ac.uk) or [peter.irving@kcl.ac.uk](mailto:peter.irving@kcl.ac.uk) (020 7188 7188 x. 82497). LORD Lab, MRC Centre for Transplantation, King’s College London, 5<sup>th</sup> Floor Tower Wing, Guy’s Hospital, London, SE1 9RT.

**Word Count: 5981**

**ABBREVIATIONS:**

- APC – antigen presenting cell
- AREG – amphiregulin
- ATP – adenosine triphosphate
- CD – Crohn’s disease
- CDAI – Crohn’s disease activity index
- CTLA-4 – cytotoxic T lymphocyte antigen 4
- DC – dendritic cell
- DR5 – death receptor 5
- DSS – dextran sulfate sodium
- Ebi3 – Epstein Barr virus induced 3
- EGF – epidermal growth factor
- FACS – fluorescence-activated cell sorting

1  
2  
3 Fgl2 – fibrinogen-like protein 2  
4  
5 Foxp3 – Forkhead box P-3  
6  
7 GALT – gut-associated lymphoid tissue  
8  
9 GC-MS – gas chromatography mass spectrometry  
10  
11 GI – gastrointestinal  
12  
13 GMP – good manufacturing practice  
14  
15 GvHD – graft versus host disease  
16  
17 HEPA – high efficiency particulate air  
18  
19 HSCT – haematopoietic stem cell transplant  
20  
21 IBD – inflammatory bowel disease  
22  
23 IFN $\gamma$  – interferon  $\gamma$   
24  
25 IL – interleukin  
26  
27 IPEX – ‘immune dysregulation, polyendocrinopathy, enteropathy, X-linked’  
28  
29 LPMC – lamina propria mononuclear cell  
30  
31 MACS – magnetic bead-activated cell sorting  
32  
33 MAdCAM-1 – mucosal vascular addressin cell adhesion molecule 1  
34  
35 MHC – major histocompatibility complex  
36  
37 MMP – matrix metalloproteinase  
38  
39 NK – natural killer  
40  
41 NOD – non-obese diabetic  
42  
43 PBMC – peripheral blood mononuclear cell  
44  
45 RAR $\alpha$  – retinoic acid receptor  $\alpha$   
46  
47 RORC – related orphan receptor C  
48  
49 RPMI – Roswell Park Memorial Institute  
50  
51 SCID – severe combined immunodeficiency  
52  
53 SLE – systemic lupus erythematosus  
54  
55 STAT – signal transducer and activator of transcription  
56  
57 T1DM – type 1 diabetes mellitus  
58  
59 TCR – T cell receptor  
60  
Teff – effector T cell  
TGF- $\beta$  – transforming growth factor  $\beta$   
Th1 – T helper 1 cell

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

Th17 – T helper 17 cell

TIGIT – T cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domains

TNBS – 2,4,6-trinitrobenzene sulfonic acid

TNF $\alpha$  – tumour necrosis factor  $\alpha$

TRAIL – tumour necrosis factor-related apoptosis inducing ligand

Treg – regulatory T cell

pTreg – peripheral regulatory T cell

tTreg – thymic regulatory T cell

UC – ulcerative colitis

**ABSTRACT:**

The prevalence of inflammatory bowel disease is rising in the Western world. Despite an increasing repertoire of therapeutic targets, a significant proportion of patients suffer chronic morbidity. Studies in mice and humans have highlighted the critical role of regulatory T cells in immune homeostasis, with defects in number and suppressive function of regulatory T cells seen in Crohn’s disease patients. We review the function of regulatory T cells and the pathways by which they exert immune tolerance in the intestinal mucosa. We explore the principles and challenges of manufacturing a cell therapy, and discuss clinical trial evidence to date for their safety and efficacy in human disease, with particular focus on the development of a regulatory T cell therapy for Crohn’s disease.

**Keywords:** Crohn’s disease, Immunology, Immunoregulation, Intestinal T cell, T lymphocytes

**INTRODUCTION:**

Inflammatory bowel disease (IBD), chiefly comprising Crohn’s disease (CD) and ulcerative colitis (UC), is a chronic inflammatory group of disorders of the gastrointestinal (GI) tract arising from overexuberant innate and adaptive immune responses to environmental factors in genetically susceptible individuals. IBD affects at least 0.5% of the population in the Western world with 1 million sufferers in USA and 2.5 million in Europe.<sup>1</sup> Global

prevalence continues to increase, largely driven by rising numbers of patients in newly industrialised regions including India and Asia.<sup>1</sup> The burden of disease is significant with 20-25% of patients experiencing chronic continuous symptoms which contributes to higher rates of unemployment, sick leave and permanent work disability.<sup>2</sup> Even with an aggressive top-down approach to therapy, the majority of patients fail to achieve prolonged, steroid-free remission and are at particular risk of requiring surgical intervention. Cumulative surgery rates in CD are high in Europe with 30-50% of patients requiring surgical intervention and up to 20% needing a reoperation 5-10 years from diagnosis.<sup>2</sup>

As our understanding of the pathophysiology of IBD and its socioeconomic impact has evolved, there has been great impetus to identify novel therapeutic targets to add to the existing arsenal of immunomodulators and biologics. These have focussed on a variety of areas including targeting lymphocyte trafficking (vedolizumab, ozanimod, anti-MAdCAM1) and activation (anti-IL6, anti-IL12/IL23), modulating intestinal barrier function (phosphatidylcholine), matrix remodelling (STNM-01, MMP9 blocker) and manipulation of gut microbiota (faecal microbiota transplant).<sup>3</sup> An important pathological process increasingly recognised as driving intestinal inflammation and autoimmunity is the loss of immune homeostasis secondary to qualitative or quantitative defects in the regulatory T cell (Treg) pool.

Tregs are CD4<sup>+</sup> T cells that characteristically express the high affinity IL-2 receptor  $\alpha$ -chain (CD25), and master transcription factor Forkhead box P-3 (Foxp3), which is essential for their suppressive phenotype and stability.<sup>4-6</sup> As activated CD4<sup>+</sup> T cells can upregulate CD25 expression, an additional defining feature of Tregs is the absence of IL-7 receptor  $\alpha$ -chain (CD127).<sup>7</sup> Their primary function is as dominant controllers of self-tolerance, tissue inflammation and long-term immune homeostasis. Despite making up only 5-10% of the peripheral CD4<sup>+</sup> T cell pool, Tregs exert powerful inhibitory effects on effector cells through a variety of mechanisms including cytokine secretion, metabolic disruption, inhibition of dendritic cells (DCs) and cytotoxicity. These mechanisms have been rigorously examined using animal models and shown to protect against the development of intestinal inflammation. Studies in patients with IBD have identified defects in the number and distribution of Tregs, and their ability to traffic to the GI tract.<sup>8</sup> Additionally, resistance to Treg-mediated

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

suppression has been noted in lamina propria T effector cells (Teffs).<sup>9</sup> These factors are likely to be pivotal in driving intestinal inflammation.

There is growing interest in the therapeutic potential of adoptively transferring healthy Tregs into patients with a wide range of conditions, including IBD and autoimmune disease, in an attempt to shift the balance in areas of active inflammation towards a more tolerogenic microenvironment. Early phase clinical trials have already reported in the fields of solid organ transplantation, graft-versus-host disease (GvHD) and type 1 diabetes mellitus (T1DM) with reassuring safety data and potential signals of efficacy.

This review provides a summary of the suppressive mechanisms utilised by Tregs and highlights seminal work linking intestinal inflammation with loss of Treg function in both animal models of disease and in humans. Additionally, we review ongoing clinical trials with Treg therapy and outline an entirely novel therapeutic strategy for CD using Tregs expanded under GMP (Good Manufacturing Practice) conditions that will be adoptively transferred to patients in an attempt to ameliorate intestinal inflammation and restore immune homeostasis.

**TREGS IN HEALTH AND DISEASE:**

Tregs can be broadly divided into two groups, thymic Tregs (tTregs) or peripherally induced Tregs (pTregs) based on their developmental origin. Tregs generated in the thymus (tTregs) in the early neonatal period migrate to peripheral organs where they maintain tolerance. This was discovered in 1969 by Nishizuka and Sakakura who showed that in mice, thymectomy 3 days after birth led to the depletion of Foxp3<sup>+</sup> Tregs and development of autoimmune oophoritis.<sup>10</sup> In contrast, mice who had thymectomy at day 7 remained healthy as the tTregs had already migrated to the periphery by this point.<sup>11</sup> Over a decade later, Sakaguchi *et al* demonstrated that day-3 thymectomy autoimmune oophoritis could be prevented with CD4<sup>+</sup> T cell inoculation from healthy syngeneic donors. Conversely, the adoptive transfer of T cells from these sick mice were capable of inducing autoimmune disease in healthy T cell deficient mice.<sup>12</sup> Similar findings were noted in rats that underwent adult thymectomy and irradiation resulting in lymphopenia, autoimmune diabetes and

insulinitis. An injection of CD45RC(low) T cells from healthy donors were capable of preventing disease.<sup>13</sup> Mottet *et al* subsequently described CD25-expressing CD4<sup>+</sup> T cells that were able to cure established T cell transfer colitis.<sup>14</sup> By the early 2000's it was clear that a thymically-derived CD4<sup>+</sup>CD25<sup>+</sup> T cell population possessed the ability to suppress autoreactive T cells and eliminate autoimmunity.

Peripherally induced Tregs (pTregs) were first described in 2003 where naïve CD4<sup>+</sup>CD25<sup>-</sup> T cells could be converted into Foxp3-expressing CD4<sup>+</sup>CD25<sup>+</sup> Tregs by T cell receptor (TCR) co-stimulation in the presence of TGF- $\beta$ .<sup>15</sup> pTreg conversion in gut-associated lymphoid tissues (GALT) was enhanced when naïve CD4<sup>+</sup> T cells encountered antigen in the presence of TGF- $\beta$ , IL-2 and retinoic acid (RA).<sup>16,17</sup> This is facilitated by CD103<sup>+</sup> DCs conditioned by the intestinal microenvironment to produce or activate TGF- $\beta$  and provide RA.<sup>18,19</sup> In the absence of CD103 expression, DCs fail to induce Treg development and produce proinflammatory cytokines.<sup>18,20</sup> Additionally, in patients with UC, CD103<sup>+</sup> DCs appear to have impaired ability to generate pTregs, but induce colitogenic T helper (Th) 1, Th2 and Th17 responses suggesting CD103<sup>+</sup> DC-mediated pTreg induction is functionally relevant in IBD pathogenesis.<sup>21</sup>

Distinguishing tTregs from pTregs can be difficult as no definitive markers exist. Recently, the expression of the membrane protein neuropilin-1 (Nrp1) and the transcription factor Helios by tTregs but not by pTregs has been used to differentiate Treg subsets.<sup>22</sup> The significance of this lies in the epigenetic differences in the *Foxp3* locus rendering pTregs less stable and more likely to demonstrate plasticity towards a Th17 cell phenotype under inflammatory conditions.<sup>23</sup> The developmental origin of Tregs selected for expansion as a cell therapy product is therefore an important consideration and will be addressed in more detail later in this review.

The first study identifying Tregs in humans was published in 2001. Baecher-Allan *et al* characterised CD4<sup>+</sup>CD25<sup>+</sup> T cells in the thymus and peripheral blood which exhibited anti-inflammatory and suppressive properties.<sup>24</sup> Subsequent work established Foxp3 as the master transcription factor for Tregs.<sup>4,6,25</sup> Foxp3 can however be expressed transiently in non-regulatory CD4<sup>+</sup> T cells upon TCR activation and the CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> surface



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

phenotype must be used to define Tregs.<sup>26</sup> Inactivating mutations in *Foxp3* clinically manifest as severe autoimmunity with a scurfy phenotype in mice and IPEX syndrome ('immune dysregulation, polyendocrinopathy, enteropathy, X-linked') in humans.<sup>27–30</sup> With autoimmune enteropathy (manifesting as chronic diarrhoea and malabsorption) a predominant feature, attention was focussed on the functional role of Tregs within the GI tract.

Peripheral Tregs are found in abundance in the intestinal lamina propria where interactions with environmental antigens can shape phenotypic differences and transcription factor expression.<sup>31</sup> The gut microbiota represents a substantial antigen load driving the expansion of colonic pTregs that co-express the Th17 master transcription factor ROR $\gamma$ t.<sup>32</sup> These Foxp3<sup>+</sup> ROR $\gamma$ t<sup>+</sup> pTregs have a stable regulatory phenotype and provide tolerance against the gut microbiota.<sup>33,34</sup> Conversely, ROR $\gamma$ t<sup>–</sup> pTregs are found in the small intestine where they are induced by dietary antigens and repress underlying Th1 cell responses to ingested proteins.<sup>35</sup> Finally, an intestinal tTreg population that co-express the Th2 master transcription factor, GATA3, has been shown to mediate repair of the intestinal mucosa. GATA3<sup>+</sup> tTregs express high levels of the IL-33 receptor, ST2, and amphiregulin, an epidermal growth factor receptor ligand involved in tissue repair.<sup>36,37</sup>

Following on from the fundamental observations linking Treg dysfunction to an array of autoimmune polyendocrine syndromes, studies began to emerge identifying defects in either number or function of peripheral blood Tregs in autoimmune disorders including IBD, type 1 diabetes, multiple sclerosis, systemic lupus erythematosus (SLE), myasthenia gravis and rheumatoid arthritis.<sup>8,38–42</sup> Maul *et al* observed that in patients with active IBD, the intestinal lamina propria Treg pool was significantly smaller than that of a positive control, namely diverticulitis.<sup>8</sup> Additionally, in these patients, the peripheral blood Treg pool was smaller than that of inactive IBD or diverticulitis.<sup>8</sup> Interestingly, the peripheral blood Tregs retained their suppressive capacity suggesting that disease may be driven by ineffective trafficking to the gut and reduced numbers of Tregs. Furthermore, colitogenic T cells from IBD patients appear to be resistant to TGF- $\beta$ 1-mediated Treg suppression highlighting an additional defect in immunological tolerance that may drive disease.<sup>43</sup>

## TREG FUNCTION AND COLITIS:

Tregs function as key mediators of peripheral tolerance through direct cellular contact and paracrine actions on tissues where they reside.<sup>44,45</sup> It is essential that Tregs effectively traffic to target organs where they promote a tolerogenic microenvironment. An important example is IL-10-secreting Tregs that reside in the GI mucosa and control inflammatory responses induced by environmental insults. Selective disruption of IL-10 expression in these Tregs has been shown to cause spontaneous colitis.<sup>46</sup> This is one of many modalities that Tregs can employ to maintain immune homeostasis at the mucosal interface. Others include inhibitory cytokine secretion, cytolysis of effector cells, metabolic disruption, neutralization of antigen presenting cells (APC) and promotion of tissue repair.<sup>47</sup> These functions will be reviewed in further detail outlining their associations with intestinal inflammation (see Figure 1).

### Inhibitory Cytokines:

The Treg cytokine repertoire includes the anti-inflammatory molecules IL-10, TGF- $\beta$  and IL-35. The expression of IL-10 and IL-35 requires TCR signalling, suggesting that Treg function in part relies on antigen encounter in the local microenvironment.<sup>48</sup> Pioneering work by Powrie *et al* over 20 years ago showcased the potent inhibitory ability of IL-10, where recombinant IL-10 therapy ameliorated established T cell transfer colitis.<sup>49</sup> Subsequently, the co-transfer of CD45RB(low) T cells were shown to prevent colitis and IL-10 was identified as an essential mediator for this *in vivo* suppression.<sup>50</sup> The suppressive effects of Treg-derived IL-10 in mice appear to be specific for mucosal surfaces rather than controlling systemic autoimmunity.<sup>46</sup> Further studies have demonstrated that IL-10 induces robust activation of a STAT3-dependent Th17 suppression program in Tregs, downstream of IL-10R.<sup>51</sup> This suppresses pathogenic Th17 cell responses and ablation of IL-10R in Tregs has been shown to cause colitis. It is therefore plausible that disordered IL-10 signalling may contribute to aberrant Th17 activity, which is implicated in IBD.<sup>52</sup> In fact, there have been several cases of homozygous loss-of-function mutations in *Il-10* and *Il-10r* arising in individuals from consanguineous marriages. These resulted in infantile severe, progressive, intractable Crohn's-like colitis.<sup>53</sup>

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

TGF- $\beta$  plays an important role inducing pTreg formation upon antigen encounter in GALT and has a functional role in suppressing pro-inflammatory pathways.<sup>54</sup> Tregs are capable of producing TGF- $\beta$ , which profoundly suppresses the proliferation of Teffs.<sup>55</sup> Treg-derived TGF- $\beta$ 1 inhibits Th1-cell differentiation and IBD in a transfer model of colitis.<sup>56</sup> Conversely, Tregs from TGF- $\beta$ 1-deficient mice fail to suppress intestinal inflammation in a SCID transfer model of colitis.<sup>55</sup> Human studies have supported these early findings; a study on healthy human colonic biopsies and lamina propria mononuclear cells (LPMC) treated with anti-TGF- $\beta$  neutralising antibody showed that TGF- $\beta$  is a critical suppressor of T-bet-dependent Teff proliferation and Th1 cytokine expression.<sup>57</sup> This suggests a role for TGF- $\beta$  in suppressing intestinal inflammation in humans. Indeed, MacDonald *et al* have shown that colonic tissue and isolated T cells from patients with CD overexpress Smad7, an inhibitor of TGF- $\beta$ 1 signalling.<sup>58</sup> Furthermore, colonic LPMCs from CD patients were resistant to Treg-mediated suppression, a phenomenon that could be reversed with Smad7 antisense treatment.<sup>43</sup> Smad 7 antisense therapy (Mongersen) was subsequently evaluated in CD but, despite promising early phase data, a phase III clinical trial was terminated early due to lack of benefit.<sup>59,60</sup> Although Mongersen may overcome Teff resistance to TGF- $\beta$ , it is possible in CD there are insufficient numbers of functional Tregs in the mucosal environment to produce TGF- $\beta$  explaining the disappointing trial outcome .

IL-35 is a heterodimer of Ebi3 and IL-12 $\alpha$  that is constitutively expressed in Foxp3<sup>+</sup> Tregs but not Teffs. It was first described in 2007 where *Ebi3*<sup>-/-</sup> and *IL-12 $\alpha$* <sup>-/-</sup> Tregs were shown to have significantly reduced regulatory activity *in vitro* and failed to cure T cell transfer colitis *in vivo*.<sup>61</sup> Additionally, IL-35 can induce the generation of a regulatory population from naïve mouse or human CD4<sup>+</sup> T cells. These so-called iT(R)35 cells mediate suppression via IL-35 alone, do not express Foxp3, and are strongly suppressive and stable *in vivo*.<sup>62</sup> In both dextran sulphate sodium (DSS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis, recombinant IL-35 therapy can treat disease through downregulation of the Th1 and Th17 master transcription factors, T-bet and RORC, respectively, and through inhibition of IFN- $\gamma$ , IL-6 and IL-17.<sup>63</sup>

**Inhibition of Metabolic Processes:**

While Tregs are not known to produce IL-2, their development and function is critically dependent on this cytokine. IL-2 and the transcription factor STAT5, downstream of IL-2 receptor (IL-2R), induce the expression of Foxp3 and differentiation of tTregs.<sup>64</sup> Furthermore, STAT5 activation driven by IL2R signalling enhances the suppressor function of differentiated Tregs.<sup>65</sup> An absence of IL-2 signalling has been shown to reduce the number and functional activity of Tregs, predisposing to autoimmunity and inflammation.<sup>66,67</sup> The structural conformation of IL-2R in Tregs provides a competitive advantage for IL-2-receptor engagement over alternative cell subsets. Tregs abundantly express IL-2 receptor  $\alpha$ -chain (CD25), which together with the common  $\gamma$ -chain ( $\gamma$ c, CD132) and IL-2 receptor  $\beta$ -chain (CD122) form a characteristic three subunit receptor configuration. This confers a ~1000-fold increase in receptor affinity for IL-2 over Teffs.<sup>68</sup> In a pro-inflammatory environment dominated by actively dividing effector cells, Tregs have the ability to “consume” local IL-2, starving effector cells of this essential cytokine for survival and proliferation.<sup>45,69</sup> Moreover, this mechanism has been shown to induce the apoptosis of effector cells.<sup>70</sup> This highlights an important TCR-independent paracrine mode of suppression in local tissues, facilitated through the constitutive expression of high affinity IL-2R (containing CD25). There have been a handful of cases of CD25 deficiency in humans often manifesting in an IPEX-like syndrome.<sup>71–73</sup> A notable case who presented with autoimmune enteropathy at 6 months had Foxp3<sup>+</sup> Tregs with defective IL-10 expression suggesting that IL-2 responsiveness is important for Treg-mediated IL-10 production.<sup>74</sup>

Tregs can also interfere with adenosine triphosphate (ATP) metabolism to dampen pro-inflammatory responses. Tregs co-express the ectoenzymes CD39 and CD73 responsible for the degradation of ATP and generation of pericellular adenosine.<sup>75</sup> Adenosine stimulates the A2A receptor on Teffs exerting potent inhibitory effects. Activation of the A2A receptor also inhibits IL-6 expression while enhancing the production of TGF- $\beta$ .<sup>76</sup> This promotes the development of adaptive induced Tregs and simultaneously inhibits pro-inflammatory Th17 cell formation. Furthermore, signalling through the A2A receptor appears to control *in vivo* murine colitis.<sup>77</sup>

#### Neutralisation of Dendritic Cell Function:

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

The activation of T cells requires TCR-antigen/MHC engagement in the context of a secondary signal, namely T cell-derived CD28 binding the DC B7 ligands, CD80 and CD86. This process is negatively regulated through the production of cytotoxic T lymphocyte antigen 4 (CTLA-4) which is constitutively expressed in Foxp3<sup>+</sup> Tregs.<sup>78</sup> CTLA-4-expressing cells can capture CD80 and CD86 by a process of trans-endocytosis and degrade these ligands, resulting in impaired co-stimulation via CD28.<sup>79</sup> This is a functionally significant process with Treg-conditioned DCs inducing poor T cell proliferation.<sup>80</sup> An additional mechanism mediated through the interaction of CTLA-4 and CD80/CD86 is the upregulation of indoleamine 2, 3-deoxygenase in DCs. This is a potent regulatory molecule which catabolises the essential amino acid tryptophan to the pro-apoptotic metabolite kynurenine leading to suppression of Teff function.<sup>64</sup> *In vivo* models have demonstrated that CTLA-4 is essential in preventing autoimmunity. Selective deletion of CTLA-4 in Tregs of BALB/c mice results in fatal T cell mediated autoimmune disease at just 20 days of age.<sup>81</sup> Additionally, several cases of germline heterozygous mutations in CTLA-4 have been identified in humans.<sup>82</sup> CTLA-4 haploinsufficiency resulted in dysregulation of Tregs, hyperactivation of Teffs and lymphocytic infiltration of target organs including the GI tract. It was recently discovered that LRBA (lipopolysaccharide-responsive and beige-like anchor protein) regulates CTLA-4 expression, where mutations in LRBA lead to reduced levels of CTLA-4.<sup>83</sup> These mutations are commonly associated with primary immunodeficiency, reduced Treg numbers and susceptibility to IBD.<sup>84,85</sup>

Recently, the coinhibitory molecule TIGIT has been described as an inhibitor of autoimmune responses through its interactions with DCs and T cells. TIGIT interacts with its ligand CD155 on DCs to induce IL-10 and suppress IL-12 production, thereby inhibiting Th1 responses.<sup>86</sup> As Tregs are the primary cell type that constitutively express TIGIT, it has been suggested that the observed effects on DCs are mediated by TIGIT<sup>+</sup> Tregs. Furthermore, Tregs expressing TIGIT have been shown to directly suppress Th1 and Th17 responses through the production of the effector molecule fibrinogen-like protein 2 (Fgl2).<sup>87</sup>

**Cytotoxic Activity:**

Historically, cytotoxic activity has been associated with natural killer (NK) cells and cytotoxic T lymphocytes (CD8<sup>+</sup> T cells). In 2004, Grossman *et al* first described granzyme-B expressing CD4<sup>+</sup> Tregs capable of killing target cells in a perforin-dependent, but TCR-independent manner.<sup>88</sup> Boissonnas *et al* subsequently showed that in a mouse tumour model, Foxp3<sup>+</sup> T cells can kill antigen-specific DCs. Treg cytotoxicity has also been observed against CD4<sup>+</sup> T cells in both *in vitro* and *in vivo* models. Activated Tregs upregulate tumour necrosis factor-related apoptosis inducing ligand (TRAIL) which enhances suppressive activity as well as cytotoxicity against CD4<sup>+</sup> T cells. This is entirely dependent on the TRAIL/death receptor 5 (DR5) pathway.<sup>89</sup> Galectin-1, a  $\beta$ -galactoside-binding protein known to induce T cell apoptosis has also been implicated in Treg cytotoxic function. Galectin-1 was found to be overexpressed in Tregs and galectin-1 knockout models were shown to possess reduced regulatory activity.<sup>90</sup>

### Tissue Repair:

Aside from limiting mucosal damage through the suppression of pro-inflammatory cells following environmental insults like infection, Tregs may also promote tissue repair. Recently, the epidermal growth factor (EGF)-like molecule amphiregulin (AREG) has gained attention as an important regulator of tissue repair and regeneration. In a murine model of influenza, selective Treg deficiency in AREG leads to severe acute lung damage without any alterations in Treg suppressor function. This suggests that Tregs play a direct role in tissue repair and maintenance that is distinct from their suppressive function.<sup>91</sup> Treg production of AREG is dependent on IL-18 or IL-33 which function as endogenous danger signals or alarmins, in response to tissue damage.<sup>91</sup> Studies in humans have revealed high levels of IL-33 in inflamed lesions of IBD patients, and Tregs expressing the IL-33 receptor, ST2, are enriched in the colon.<sup>92–94</sup> IL-33-Treg signalling may therefore represent an important pathway in both disease pathogenesis and recovery.

### TREGS AS A THERAPEUTIC PRODUCT:

In light of the vast array of preclinical data showcasing how a multitude of defects in Treg function contribute to autoimmunity and inflammation, including IBD, there has been great

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

interest in harnessing the suppressive ability of Tregs as a therapeutic product. Consequently, there are over 50 registered trials of Treg therapy that are either completed or ongoing (clinicaltrials.gov). Most of these trials involve adoptive cell transfer, although the dose of Tregs given is highly variable. In the setting of autoimmune disease and transplantation, the goals of treatment are the restoration of peripheral self tolerance, the suppression of inflammation and promotion of tissue repair.<sup>95</sup>

In order to become a successful therapeutic product, Tregs must home to sites of inflammation and secondary lymphoid tissues, and must undergo TCR engagement. It has been demonstrated in solid organ transplantation that alloantigen-specific Tregs provide higher therapeutic benefits than polyclonal Tregs, without delivering a systemic immunosuppressive effect.<sup>96</sup> Directing Tregs against a specific alloantigen also permits immunomodulatory functions to be concentrated at the site of the alloantigen source, circumventing the relative paucity of Tregs. An early study demonstrated that peripheral Treg expansion in mice could be driven by prolonged low dose subcutaneous infusion of a specific peptide.<sup>97</sup> The induced Tregs had suppressive abilities, and demonstrated high levels of Foxp3 expression indicating a stable Treg phenotype. However, in IBD, a specific antigen has yet to be identified.

The relative paucity of Tregs in peripheral blood represents an obstacle to the development of a cellular therapy, though the optimum number of Tregs to be infused remains unclear. It has been suggested that the number of Tregs given should be at least as great as the number of T cells in the body,<sup>98</sup> though Tregs also exhibit the ability to confer suppressive ability on conventional T cells through ‘infectious tolerance’.<sup>96</sup> In this process, the direct secretion of TGF- $\beta$ , IL-10 and IL-35 by Tregs, and indirect induction via DCs, can generate a regulatory microenvironment which may partially circumvent the problem of low absolute numbers of Tregs.<sup>99</sup>

Several groups have developed protocols in line with GMP requirements to permit *ex vivo* cell expansion of Tregs.<sup>98,100,101</sup> GMP-manufactured Tregs delivered in some early trials were only around 50% pure, but the development of plastic beads coated with stimulatory antibodies and the discovery of additional surface markers for Treg phenotyping mean that



1  
2  
3 a product with purity greater than 90% is now achievable.<sup>98</sup> Contamination of the expansion  
4 product with Teffs hampers expansion,<sup>102</sup> but the inclusion of rapamycin in cell culture  
5 blocks expansion of Teffs without affecting Treg proliferation, leading to the preferential  
6 promotion of Treg proliferation.<sup>98,103</sup>  
7  
8  
9

10  
11  
12 Tregs are first isolated from peripheral blood by surface marker expression  
13 (CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup>). This can be performed using stream in air fluorescence-activated cell  
14 sorters (FACS) which yield a highly pure starting population, but the necessary air exposure  
15 requires high efficiency particulate air (HEPA) enclosures, and single use sample lines to be  
16 compatible with manufacturing GMP cell products. Closed system magnetic bead-activated  
17 cell sorting (MACS) can be adapted for large-scale isolation of human Tregs, but unlike FACS  
18 cannot easily distinguish surface marker expression density. A recently developed  
19 microfluidic chip fluorescence-activated cell sorter, the MACSQuant Tyto (Miltenyi Biotech,  
20 Germany) surmounts the problems of stream in air sorters, as the cells remain in a closed  
21 system throughout the sorting process. Expansion of the sorted cells is achieved through  
22 polyclonal TCR activation with anti-CD3/anti-CD28 beads.<sup>104</sup> Tregs are sampled and checked  
23 for sterility and phenotype throughout the expansion process. With optimised conditions, a  
24 500-fold expansion can be anticipated over a 14 day period.<sup>101</sup>  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37

38 Uncertainty about the plasticity of Tregs in culture and following infusion means there is a  
39 theoretical concern about the development of a pro-inflammatory phenotype, which could  
40 lead to transplant rejection or aggravation of inflammation. However, rapamycin-expanded  
41 Tregs are not contaminated by IL-17-producing Th17 cells, and these cells maintain a stable  
42 phenotype on transfer *in vivo* to mice.<sup>105</sup> Canavan *et al.* found that the starting population  
43 for Treg expansion from the peripheral blood of CD patients has a critical effect on the  
44 phenotype of the expanded cell population.<sup>100</sup> Tregs from a highly pure FACS-sorted 'naïve'  
45 CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup>CD45RA<sup>+</sup> precursor population demonstrated enhanced suppressive  
46 ability and reduced Th17 plasticity *in vitro* compared to a FACS-sorted  
47 CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup>CD45RA<sup>-</sup> or MACS-enriched CD8<sup>-</sup>CD25<sup>+</sup> population. Rapamycin appears to  
48 imprint a fixed CD4<sup>+</sup>CD25<sup>hi</sup> phenotype to cells expanded from a 'naïve' CD45RA<sup>+</sup> population,  
49 as evidenced by the retention of demethylation at the Foxp3 locus.  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60



TREG THERAPY IN OTHER CONDITIONS:

There is an increasing body of evidence for the use of Tregs as cellular therapy in autoimmune disease and transplantation (see Table 1). Adoptive transfer of Tregs to prevent GvHD was the first illustration of the potent therapeutic potential of Tregs in experimental transplantation.

Study	Clinical context	Enrichment protocol	Expansion protocol	Dose	Study outcome
Trzonkowski <i>et al.</i> (2009)	Treatment of acute and chronic GvHD N=2	Tregs from allogenic buffy coat. CD4 <sup>+</sup> negative bead selection followed by FACS-based sorting of CD4 <sup>+</sup> CD25 <sup>hi</sup> CD127 <sup>lo</sup> cells	RPMP 1640 with 10% autologous plasma IL-2 (1000IU/ml) Anti-CD3/anti-CD28 beads (1:1) 3 weeks	Acute GvHD: 1x10 <sup>6</sup> /kg Chronic GvHD: 3x10 <sup>6</sup> /kg	Transient improvement in acute GvHD; alleviation of symptoms and reduction of immunosuppression in chronic GvHD
Brunstein <i>et al.</i> (2011)	Prevention of GvHD following umbilical cord blood transplantation N=23	CD25 <sup>+</sup> bead positive selection	X-Vivo 15 with 10% human AB serum IL-2 (300IU/ml) Anti-CD3/anti-CD28 beads (1:2) 18±1 days	0.1-30x10 <sup>5</sup> /kg	Well –tolerated; reduced incidence of grade II-IV GvHD in Treg recipients
Marek-Trzonkowska <i>et al.</i> (2012)	Safety of autologous <i>in vitro</i> expanded Tregs in paediatric type 1 diabetes N=10	FACS-based sorting of CD3 <sup>+</sup> CD4 <sup>+</sup> CD25 <sup>hi</sup> CD127 <sup>lo</sup> cells	CellGro medium with 10% autologous plasma IL-2 (1000IU/ml) Anti-CD3/anti-CD28 beads (1:1) Up to 2 weeks	10-20x10 <sup>6</sup> /kg	Well-tolerated; decreased insulin requirements and C-peptide levels in Treg recipients
Desreumaux <i>et al.</i> (2012)	Safety and efficacy in Crohn’s disease N=20	Culture of PBMCs with ovalbumin, IL-2 and IL-4 followed by cloning of ovalbumin-specific T cells	X-Vivo 15 IL-2 (200IU/ml) Anti-CD3/anti-CD28 beads (1:1) Ova-Tregs selected based on ovalbumin-specific IL-10 production 12 to 15 weeks	1x10 <sup>6</sup> -1x10 <sup>9</sup>	Well-tolerated; dose-related efficacy
Bluestone <i>et al.</i> (2015)	Safety in adults with type 1 diabetes (N=14)	FACS-based sorting of CD4 <sup>+</sup> CD25 <sup>hi</sup> CD127 <sup>lo</sup> cells	X-Vivo 15 with 10% human AB serum and deuterated glucose IL-2 (300IU/ml) Anti-CD3/anti-CD28 beads (1:1) 14 days	0.05x10 <sup>8</sup> -26x10 <sup>8</sup>	Well-tolerated, no significant adverse events. Stable C-peptide levels and insulin use in recipients for up to two years post infusion
Mathew <i>et al.</i> (2018)	Safety in living donor kidney transplant N=9	CliniMACS plus GMP enrichment system (Miltenyi)	IL-2 (1000IU/ml) MACS © GMP expansion beads 1:1-4:1 3 weeks	0.5-5x10 <sup>9</sup>	Well-tolerated, no infections or rejection up to two years post transplant

**Table 1: Summary of clinicaltrials.gov listings for reported trials using *in vitro* expanded regulatory T cell (Treg) therapy**

**Graft vs. host disease (GvHD):**

The risk of developing GvHD following haematopoietic stem cell transplantation (HSCT) is associated with low numbers of Tregs in the periphery,<sup>106</sup> and *in vivo* expansion of Tregs post-HSCT using low dose IL-2 has demonstrated efficacy against GvHD.<sup>107,108</sup> Studies in mice involving infusion of cultured CD4<sup>+</sup>CD25<sup>+</sup> T cells resulted in a significantly reduced GvHD phenotype,<sup>109</sup> and in humans it was found that infusion of freshly isolated donor Tregs given at the same time as haplotype mismatched HSCT prevented the development of GvHD.<sup>110</sup> Five trials of *ex vivo* expanded Tregs have to date involved small numbers of patients only, but suggest therapy can prevent or delay the onset of chronic GvHD<sup>111,112</sup>. Treg therapy seems to be effective only in the chronic form of GvHD, but this may be because of the time requirements to expand the cellular product which makes it difficult to administer in a timely manner in acute GvHD.<sup>113</sup>

**Solid organ transplant:**

Adoptive Treg therapy has been trialled following renal and liver transplantation, with the aim of inducing tolerance to the allograft and reducing the burden of long-term immunosuppression.<sup>114</sup> Tregs have been shown to control immune responsiveness to alloantigens and contribute to 'operational tolerance' in preclinical transplantation models.<sup>115,116</sup> Recipient-derived Tregs expanded for direct and indirect pathway allospecificity *in vitro* were able to mediate effective protection against acute and chronic rejection in skin and heart allografts in mice,<sup>117</sup> and could be used to induce tolerance of a murine skin transplant following thymectomy and T cell depletion.<sup>118</sup> In these models, alloantigen reactive Tregs were more effective at preventing graft rejection than polyclonally expanded Tregs<sup>104</sup>.

A phase I study in renal transplantation recruited nine living donor transplant recipients, and used the product of leukapheresis as the basis for *ex vivo* expansion of polyclonal

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

autologous Tregs.<sup>114</sup> Alemtuzumab was given at induction to achieve lymphodepletion, on the basis of previous experiments suggesting a reduction in circulating Tregs worked synergistically with Treg infusion to prolong allograft survival.<sup>116</sup> Recipients were switched from traditional immunosuppression with tacrolimus, which blocks IL-2 production, to sirolimus (rapamycin), which has Treg promoting activity.<sup>119</sup>

An enhanced suppressive ability of the expanded Tregs was demonstrated when compared to Tregs taken directly *ex vivo*.<sup>114</sup> There were no adverse infusion-related side effects, infections or rejection up to two years post-transplant, and there was a 5-20 fold increase in the number of circulating Tregs seen up to one year post-transplant. Transplant biopsies taken at three months did not show rejection and recipients had not developed peripheral donor-specific antibodies. An additional important outcome from trials in transplantation is that they have demonstrated that it is possible to expand Tregs from immunocompromised patients.<sup>120</sup>

A trial of Treg immunotherapy in liver transplantation is currently underway.<sup>121</sup> This is predicated on the observation that when liver allografts in mice were infiltrated with Tregs, loss of Treg numbers was associated with a loss of tolerance.<sup>122</sup> Increased frequencies of Tregs are also seen in human subjects who acquire ‘operational tolerance’ to their liver transplant.<sup>123</sup>

**Type 1 diabetes mellitus:**

The development of T1DM is associated with deficits in the number and suppressive activity of Tregs.<sup>124</sup> Accelerated diabetes onset is seen in both scurfy mice<sup>29</sup> and children with IPEX,<sup>125</sup> highlighting the role of Tregs in protecting pancreatic islet cells from destruction. Tregs have been implicated in the pathogenesis of diabetes in the non-obese diabetic (NOD) mouse model,<sup>126,127</sup> and anti-CD3 antibodies have been efficacious in the treatment of diabetes in both mouse<sup>128,129</sup> and human trials.<sup>130,131</sup> Subjects exhibited lower insulin requirements and higher C-peptide levels at least 18 months after a short course of intravenous treatment, with evidence of anti-CD3 treatment inducing expansion of a CD4<sup>+</sup>CD25<sup>+</sup> T cell population.<sup>129</sup> A trial of ten children treated with expanded polyclonal Tregs

1  
2  
3 within two months of their diagnosis demonstrated statistically lower insulin requirements  
4 and C peptide levels compared with matched controls up to six months post infusion, with  
5 two patients remaining insulin-independent.<sup>124</sup> There were no serious adverse events up to  
6 one year following infusion.  
7  
8  
9

10  
11  
12 In a phase I open-label trial of 14 adult patients infused with *ex vivo* expanded Tregs in  
13 escalating doses, 7 of 14 patients had stable C peptide levels and insulin use for up to two  
14 years following infusion.<sup>101</sup> However, the study was not powered to detect significant  
15 clinical improvement. There were no infusion reactions or therapy-related serious adverse  
16 events. Phenotypic analysis of the cell product after expansion and after infusion identified  
17 stable surface marker expression, demonstrating that the infused Tregs did not acquire a  
18 pathological phenotype. High throughput TCR- $\beta$  sequencing analysis indicated that  
19 expanded Tregs retained a high degree of diversity.  
20  
21  
22  
23  
24  
25  
26  
27  
28

29 Adoptively transferred Tregs were tagged by labelling the deoxyribose moiety of replicating  
30 DNA during expansion *ex vivo*, through addition of deuterated [6,6-<sup>2</sup>H<sub>2</sub>] glucose to Treg  
31 culture throughout the 14 day expansion period.<sup>101</sup> Patient samples were analysed by gas  
32 chromatography mass spectrometry (GC-MS) for deuterium enrichment to create  
33 pharmacokinetic curves. Adoptively transferred T cell numbers peaked at two weeks  
34 following infusion, but were still detectable at up to 25% of the peak level at one year in  
35 peripheral blood. Significantly, deuterium labelling was never found in non-Tregs, indicating  
36 the stability of infused Tregs. However, due to the nature of this study, the stability of these  
37 cells was not assessed within the target tissue.  
38  
39  
40  
41  
42  
43  
44  
45  
46

#### 47 **TREG THERAPY IN INFLAMMATORY BOWEL DISEASE:**

48  
49  
50

51 A local imbalance between Treg and Teff responses plays a key role in the development of  
52 gut inflammation in IBD.<sup>8</sup> T cell gut homing is mediated by specific interaction between  
53 integrin  $\alpha 4\beta 7$  and its ligand MAdCAM-1.<sup>132,133</sup> Several groups have shown that transfer of  
54 Tregs into mice leads to clinical and histological improvement in colitis,<sup>14,134,135</sup> and  
55 rapamycin-expanded Tregs ameliorated established colitis in a SCID mouse model.<sup>136</sup>  
56 Polyclonality of the TCR is likely to be an important requirement for Tregs to maintain  
57  
58  
59  
60

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

intestinal homeostasis *in vivo*. Mice which express a restricted TCR repertoire develop spontaneous colitis due to a loss of tolerance to intestinal microbiota.<sup>137</sup>

Several groups have demonstrated that it is feasible to extract Tregs from patients, and expand them *in vitro* under GMP conditions, including from subjects receiving thiopurines and anti-TNF $\alpha$  medications.<sup>98,100,103,138</sup> Even after prolonged culture, these Tregs maintained Foxp3 expression and demonstrated enhanced suppression of autologous T cells. Uncertainty regarding the potential for adoptively transferred Tregs to express IL-17 and exacerbate CD lesions is a concern. However, the administration of pro-inflammatory cytokines (IL-1, IL-2, IL-6, IL-21, IL-21 and TGF- $\beta$ ) failed to induce IL-17 production by CD45RA<sup>+</sup> expanded Tregs *in vitro*.<sup>100</sup>

**Antigen specific vs. polyclonal Treg cell products for Crohn’s disease:**

No antigens have yet been verified as causal in CD. Attempts have been made to identify shared TCRs between CD sufferers with the aim of discovering target antigens.<sup>139–141</sup> This work has observed that the CD4 TCR repertoires are significantly more diverse in patients with CD and UC than healthy controls.<sup>142</sup> This may be explained by GI barrier disruption increasing the number of antigen presentation events in comparison to a healthy gut. Resolving a target from the GI peptidome is challenging due to the heterogenous nature of the environment. Developments in the understanding of non-conventional epitopes are also increasing the magnitude and complexity of the peptidome itself.<sup>143</sup> In the absence of a known target, the broad reactivity of a polyclonal Treg product may be advantageous, as the cell product will recognise millions of putative epitopes, increasing the likelihood of TCR engagement and subsequent Treg activation. Sequencing of isolated Tregs from GI biopsies post transfer may yield novel targets, upon which chimeric antigen receptor technology could be readily implemented.<sup>144</sup>

For Treg therapy to be effective in IBD, expanded Tregs must have the ability to home to the gut.<sup>145</sup> A French group reported the results of an open label multicentre phase I/IIa trial of ovalbumin-specific Tregs in 20 patients with refractory CD.<sup>146</sup> Ovalbumin is a common food antigen, and is not implicated in intestinal inflammation in animal models or in patients with

CD. Its distribution along the digestive tract can be used to activate Tregs locally. In the study, this was facilitated through ingestion of meringue cakes by subjects.<sup>146</sup>

The cell product was cultured in the presence of ovalbumin, and trial subjects received a dose of  $10^6$ - $10^9$  Tregs.<sup>146</sup> Patients enrolled in the study had at least moderately active CD, with a Crohn's Disease Activity Index (CDAI) greater than or equal to 220 within six months of screening, and a washout period was required for immunosuppression and anti-TNF $\alpha$  therapy. The infusion was well tolerated, with mild GI symptoms and CD flares being the most commonly reported adverse effects. Two patients experienced thrombotic events, but these are known to occur more frequently in inflammatory conditions including active CD.<sup>147</sup> Eight (40%) patients had a significant CDAI response at weeks five and eight after treatment, with two patients experiencing sustained remission. Overall, the results suggested good tolerability in this disease group with possible signals of efficacy.

In the absence of a known antigen, other methods must be used to direct the Tregs to the areas of inflammation. A recent study has shown that a highly specific retinoic acid receptor  $\alpha$  (RAR $\alpha$ ) agonist induces expression of Integrin  $\alpha 4\beta 7$  (the ligand of MAdCAM-1) on the Treg surface. Adoptive transfer of RAR $\alpha$  agonist-treated Tregs leads to improved Treg trafficking to gut tissue in a humanised mouse model of colitis.<sup>100</sup> Supporting this mechanism for resolving inflammation, another group have demonstrated that DCs can be engineered *de novo* to produce high concentrations of RA.<sup>148</sup> When transferred to mice, the RA-secreting DCs were able to augment the expression of Foxp3 and the gut-homing receptor CCR9 in native Tregs with the subsequent suppression of colitis.

The RAR $\alpha$  agonist treated cell product forms the basis of the TRIBUTE trial (ClinicalTrials.gov Identifier: NCT03185000), a double-blinded placebo-controlled phase I/IIa trial of adoptive Treg therapy in CD.

#### **FUTURE DEVELOPMENTS IN TREG THERAPY:**

The potential therapeutic benefits of adoptive cell therapy are being explored in numerous autoimmune conditions. In SLE, adoptive transfer of *ex vivo* expanded Tregs in mice delayed

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

the onset of renal complications and prolonged survival,<sup>149,150</sup> and a pilot study of low dose IL-2 in 37 patients led to increased circulating peripheral Treg numbers and decreased SLE disease activity scores.<sup>151</sup> Adoptive Treg transfer in a single patient with cutaneous lupus did not lead to clinical benefit, but increased percentages of highly activated Tregs were identified in biopsies taken from diseased skin.<sup>152</sup> Treg accumulation in skin was associated with a marked attenuation of IFN- $\gamma$ , which was more pronounced relative to peripheral blood.

Preliminary results from mouse models suggest a role for Treg therapy in conditions as diverse as pemphigus vulgaris,<sup>153</sup> autoimmune hepatitis,<sup>154</sup> multiple sclerosis,<sup>113</sup> asthma, and allergy, in which antigen-specific Tregs may represent a viable therapeutic option.<sup>155,156</sup>

Many ongoing challenges exist for the advancement of Treg therapy. Uncertainties remain about the optimal timing of *ex vivo* Treg expansion, and whether IL-2 administration would be a useful adjunct to support a Treg population *in vivo*.<sup>101,107</sup> In addition, concomitant treatment of autoimmune disease with immunosuppressive drugs may affect the function of adoptively transferred cells.<sup>95</sup>

The optimal dosing strategy for Treg therapy also remains unclear, although data tracking the survival of deuterium-labelled Tregs *in vivo* could be invaluable in informing a suitable dosing regimen.<sup>101</sup> A two-phase decay in numbers of deuterium-labelled Tregs has been seen, with 75% of the peak level lost at three months. However, levels stabilised at one year, with up to 25% of peak Treg numbers remaining in the peripheral circulation. The decrease in labelled Tregs may represent cell death, trafficking to lymphoid tissue and sites of inflammation, or proliferation of the Treg compartment leading to dilution of deuterium enrichment. Reassuringly, at no point during the trial was deuterium detected in cell populations other than Tregs, suggesting a stable phenotype *in vivo*.<sup>101</sup>

Tracking of TCR clonotypes may also provide useful data on Treg kinetics and dispersal. Analysis of the TCR repertoire has suggested that the kinetics of transferred Tregs in peripheral blood varies significantly between individuals<sup>157</sup>. In a descriptive study, the TCR V $\alpha$  chain was sequenced in two patients receiving donor Treg infusion<sup>157</sup>. Treg therapy



1  
2  
3 altered the patients' peripheral TCR repertoire considerably towards that of the infused cell  
4 product, but to different degrees in each patient. Importantly, the degree of alteration of  
5 the TCR repertoire appeared to correlate with clinical response. This suggests that  
6 monitoring TCR repertoires following adoptive cell transfer may provide clinically  
7 meaningful information.  
8  
9  
10  
11  
12  
13  
14  
15

## 16 **CONCLUSION:**

17  
18  
19  
20 There is now robust evidence of the therapeutic potential of Treg therapy in Crohn's  
21 disease. Trials in multiple autoimmune diseases and results from use of ovalbumin-specific  
22 Tregs in IBD show promising early signs of efficacy. The safety signal is reassuring, with  
23 evidence that the adoptively transferred Treg phenotype is stable *in vivo*. Results from  
24 deuterium labelling suggest that infused Tregs may be able to exert a long-lasting systemic  
25 effect with labelled cells detectable up to a year after infusion. It is hoped that upcoming  
26 early phase clinical trials in patients with Crohn's disease will inform safety, dosing, and Treg  
27 kinetics and dispersal allowing further development of a novel therapeutic option in this  
28 hard-to-treat population.  
29  
30  
31  
32  
33  
34  
35  
36  
37

## 38 **FIGURE LEGEND:**

### 39 **Figure 1. Mechanisms of Treg mediated suppression**

40  
41  
42 Tregs utilize a multitude of mechanisms to promote a tolerogenic microenvironment and  
43 tissue repair. (A) Secretion of the anti-inflammatory cytokines, IL-10, TGF- $\beta$  and IL-35, not  
44 only inhibit Teff proliferation, but also suppress Th1 and Th17 effector function, both of  
45 which are key mediators of IBD. (B) Tregs express the high affinity IL-2 receptor  $\alpha$ -chain  
46 (CD25) consuming local IL-2 with greater affinity than effector cells. Teffs which are 'starved'  
47 of IL-2 exhibit restricted proliferation and undergo apoptosis. (C) Tregs co-expressing CD39  
48 and CD73 disrupt metabolic processes in effector cells by converting ATP into peri-cellular  
49 adenosine, a potent inhibitor of Teff function. Additionally, adenosine stimulates TGF- $\beta$   
50 production, promoting development of pTregs. (D) Tregs are capable of secreting perforin,  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

granzyme B and galectin-1 which are directly cytotoxic against Teffs. Activated Tregs also express TRAIL, inducing apoptosis of Teffs through the TRAIL/DR5 pathway. (E) Expression of CTLA-4 degrades DC-derived CD80 and CD86 leading to impaired CD28-mediated co-stimulation of T cells. DC function is further inhibited through the interaction of Treg-derived TIGIT and CD155 on DCs. This induces IL-10 production and suppresses IL-12. (F) In response to alarmins, Tregs produce AREG, an important regulator of tissue repair and regeneration. AREG, amphiregulin; ATP, adenosine tri-phosphate; CTLA-4, cytotoxic T lymphocyte associated protein 4; DC, dendritic cell; DR5, death receptor 5; IBD, inflammatory bowel disease; pTregs, peripheral regulatory T cells; Teff, T effector lymphocyte; TGF- $\beta$ , transforming growth factor beta; Th1, T helper 1 cell; Th17, T helper 17 cell; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; TRAIL, TNF-related apoptosis-inducing ligand; Treg, regulatory T cell. Figure generated using BioRender© illustration software.

**ACKNOWLEDGEMENTS:**

Support was provided by Litwin IBD Pioneers Funding Program at the Crohn’s and Colitis Foundation (grant number 504039), the Freemason’s Grand Charity (grant number SPG00268) and the Rosetree’s Trust (A1219). OO is funded by an MRC Trust Clinical Research Training Fellowship (MRS00033X/1). This work was also supported by MRC Grants to GL (MR/R001413/1) and to PI (MR/T005564/1). The authors also wish to acknowledge financial support from the Department of Health via the National Institute for Health (NIHR) comprehensive Biomedical Research Centre award to Guy’s & St Thomas’ NHS Foundation Trust in partnership with King’s College London and King’s College Hospital NHS Foundation Trust. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health.

**COMPETING INTERESTS:**

PI is the Chief Investigator and GL is the Chief Scientific Investigator on the MRC-funded TRIBUTE trial of regulatory T cell immunotherapy in Crohn’s disease (ClinicalTrials.gov NCT03185000).

Confidential: For Review Only

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

## References:

1. Kaplan, G. G. The global burden of IBD: from 2015 to 2025. *Nat. Rev. Gastroenterol. Hepatol.* **12**, 720–727 (2015).
2. Burisch, J., Jess, T., Martinato, M., Lakatos, P. L. & ECCO -EpiCom. The burden of inflammatory bowel disease in Europe. *J. Crohn's Colitis* **7**, 322–337 (2013).
3. Neurath, M. F. Current and emerging therapeutic targets for IBD. *Nat. Rev. Gastroenterol. Hepatol.* **14**, 269–278 (2017).
4. Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat. Immunol.* **4**, 330–336 (2003).
5. Hori, S., Nomura, T. & Sakaguchi, S. Control of Regulatory T Cell Development by the Transcription Factor Foxp3. *Science* (80-. ). **299**, 1057–1061 (2003).
6. Khattari, R., Cox, T., Yasayko, S.-A. & Ramsdell, F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat. Immunol.* **4**, 337–342 (2003).
7. Liu, W. *et al.* CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J. Exp. Med.* **203**, 1701–11 (2006).
8. Maul, J. *et al.* Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease. *Gastroenterology* **128**, 1868–78 (2005).
9. Fantini, M. C. *et al.* Smad7 Controls Resistance of Colitogenic T Cells to Regulatory T Cell-Mediated Suppression. *Gastroenterology* **136**, 1308–1316.e3 (2009).
10. Nishizuka, Y. & Sakakura, T. Thymus and reproduction: sex-linked dysgenesis of the gonad after neonatal thymectomy in mice. *Science* **166**, 753–5 (1969).
11. Sakaguchi, S., Takahashi, T. & Nishizuka, Y. Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis. *J. Exp. Med.* **156**, 1577–86 (1982).
12. Sakaguchi, S., Takahashi, T. & Nishizuka, Y. Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis. *J. Exp. Med.* **156**, 1577–86 (1982).
13. Fowell, D. & Mason, D. Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. Characterization of the CD4+ T cell subset that inhibits this autoimmune potential. *J. Exp. Med.* **177**, 627–36 (1993).
14. Mottet, C., Uhlig, H. H. & Powrie, F. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. *J. Immunol.* **170**, 3939–43 (2003).
15. Chen, W. *et al.* Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J. Exp. Med.* **198**, 1875–86 (2003).
16. Sun, C.-M. *et al.* Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J. Exp. Med.* **204**, 1775–85 (2007).
17. Kanamori, M., Nakatsukasa, H., Okada, M., Lu, Q. & Yoshimura, A. Induced Regulatory T Cells: Their Development, Stability, and Applications. *Trends Immunol.* **37**, 803–811 (2016).
18. Coombes, J. L. *et al.* A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J. Exp. Med.* **204**, 1757–64 (2007).
19. Ruane, D. T. & Lavelle, E. C. The Role of CD103+ Dendritic Cells in the Intestinal Mucosal Immune System. *Front. Immunol.* **2**, 25 (2011).
20. del Rio, M.-L., Bernhardt, G., Rodriguez-Barbosa, J.-I. & Förster, R. Development and

- functional specialization of CD103<sup>+</sup> dendritic cells. *Immunol. Rev.* **234**, 268–281 (2010).
21. Matsuno, H. *et al.* CD103<sup>+</sup> Dendritic Cell Function Is Altered in the Colons of Patients with Ulcerative Colitis. *Inflamm. Bowel Dis.* **23**, 1524–1534 (2017).
  22. Nutsch, K. *et al.* Rapid and Efficient Generation of Regulatory T Cells to Commensal Antigens in the Periphery. *Cell Rep.* **17**, 206–220 (2016).
  23. Kanamori, M., Nakatsukasa, H., Okada, M., Lu, Q. & Yoshimura, A. Induced Regulatory T Cells: Their Development, Stability, and Applications. *Trends Immunol.* **37**, 803–811 (2016).
  24. Baecher-Allan, C., Brown, J. A., Freeman, G. J. & Hafler, D. A. CD4<sup>+</sup>CD25<sup>high</sup> regulatory cells in human peripheral blood. *J. Immunol.* **167**, 1245–53 (2001).
  25. Gavin, M. A. *et al.* Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* **445**, 771–775 (2007).
  26. Martin, F., Ladoire, S., Mignot, G., Apetoh, L. & Ghiringhelli, F. Human FOXP3 and cancer. *Oncogene* **29**, 4121–4129 (2010).
  27. Brunkow, M. E. *et al.* Disruption of a new forkhead/winged-helix protein, scurf, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* **27**, 68–73 (2001).
  28. Bennett, C. L. *et al.* The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* **27**, 20–21 (2001).
  29. Wildin, R. S. *et al.* X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat. Genet.* **27**, 18–20 (2001).
  30. Husebye, E. S., Anderson, M. S. & Kämpe, O. Autoimmune Polyendocrine Syndromes. *N. Engl. J. Med.* **378**, 1132–1141 (2018).
  31. Weiss, J. M. *et al.* Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3<sup>+</sup> T reg cells. *J. Exp. Med.* **209**, 1723 (2012).
  32. Sefik, E. *et al.* Individual intestinal symbionts induce a distinct population of RORγ<sup>+</sup> regulatory T cells. *Science (80-. )*. **349**, 993–7 (2015).
  33. Yang, B.-H. *et al.* Foxp3<sup>+</sup> T cells expressing RORγt represent a stable regulatory T-cell effector lineage with enhanced suppressive capacity during intestinal inflammation. *Mucosal Immunol.* **9**, 444–457 (2016).
  34. Whibley, N., Tucci, A. & Powrie, F. Regulatory T cell adaptation in the intestine and skin. *Nature Immunology* **20**, 386–396 (2019).
  35. Kim, K. S. *et al.* Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine. *Science (80-. )*. **351**, 858–863 (2016).
  36. Ohnmacht, C. *et al.* MUCOSAL IMMUNOLOGY. The microbiota regulates type 2 immunity through RORγt<sup>+</sup> T cells. *Science* **349**, 989–93 (2015).
  37. Schiering, C. *et al.* The alarmin IL-33 promotes regulatory T-cell function in the intestine. *Nature* **513**, 564–568 (2014).
  38. Lindley, S. *et al.* Defective suppressor function in CD4<sup>(+)</sup>CD25<sup>(+)</sup> T-cells from patients with type 1 diabetes. *Diabetes* **54**, 92–9 (2005).
  39. Viglietta, V., Baecher-Allan, C., Weiner, H. L. & Hafler, D. A. Loss of functional suppression by CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in patients with multiple sclerosis. *J. Exp. Med.* **199**, 971–9 (2004).

40. Bonelli, M. *et al.* Quantitative and qualitative deficiencies of regulatory T cells in patients with systemic lupus erythematosus (SLE). *Int. Immunol.* **20**, 861–868 (2008).
41. Thiruppathi, M. *et al.* Functional defect in regulatory T cells in myasthenia gravis. *Ann. N. Y. Acad. Sci.* **1274**, 68–76 (2012).
42. van Roon, J. A. G., Hartgring, S. A. Y., van der Wurff-Jacobs, K. M. G., Bijlsma, J. W. J. & Lafeber, F. P. J. G. Numbers of CD25+Foxp3+ T cells that lack the IL-7 receptor are increased intra-articularly and have impaired suppressive function in RA patients. *Rheumatology* **49**, 2084–2089 (2010).
43. Fantini, M. C. *et al.* Smad7 Controls Resistance of Colitogenic T Cells to Regulatory T Cell-Mediated Suppression. *Gastroenterology* **136**, 1308–1316.e3 (2009).
44. Takahashi, T. *et al.* Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int. Immunol.* **10**, 1969–80 (1998).
45. Thornton, A. M. & Shevach, E. M. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* **188**, 287–96 (1998).
46. Rubtsov, Y. P. *et al.* Regulatory T Cell-Derived Interleukin-10 Limits Inflammation at Environmental Interfaces. *Immunity* **28**, 546–558 (2008).
47. Vignali, D. A. A., Collison, L. W. & Workman, C. J. How regulatory T cells work. *Nat. Rev. Immunol.* **8**, 523–532 (2008).
48. Levine, A. G., Arvey, A., Jin, W. & Rudensky, A. Y. Continuous requirement for the TCR in regulatory T cell function. *Nat. Immunol.* **15**, 1070–1078 (2014).
49. Powrie, F. *et al.* Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity* **1**, 553–62 (1994).
50. Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L. & Powrie, F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* **190**, 995–1004 (1999).
51. Chaudhry, A. *et al.* Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity* **34**, 566–78 (2011).
52. Ahern, P. P., Izcue, A., Maloy, K. J. & Powrie, F. The interleukin-23 axis in intestinal inflammation. *Immunol. Rev.* **226**, 147–159 (2008).
53. Engelhardt, K. R. & Grimbacher, B. IL-10 in humans: Lessons from the Gut, IL-10/IL-10 receptor deficiencies, and IL-10 polymorphisms. *Curr. Top. Microbiol. Immunol.* **380**, 1–18 (2014).
54. Sun, C.-M. *et al.* Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J. Exp. Med.* **204**, 1775–85 (2007).
55. Nakamura, K. *et al.* TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice. *J. Immunol.* **172**, 834–42 (2004).
56. Li, M. O., Wan, Y. Y. & Flavell, R. A. T Cell-Produced Transforming Growth Factor-β1 Controls T Cell Tolerance and Regulates Th1- and Th17-Cell Differentiation. *Immunity* **26**, 579–591 (2007).
57. Di Sabatino, A. *et al.* Blockade of transforming growth factor beta upregulates T-box transcription factor T-bet, and increases T helper cell type 1 cytokine and matrix metalloproteinase-3 production in the human gut mucosa. *Gut* **57**, 605–12 (2008).
58. Monteleone, G. *et al.* Blocking Smad7 restores TGF-beta1 signaling in chronic inflammatory bowel disease. *J. Clin. Invest.* **108**, 601–9 (2001).

59. Monteleone, G. *et al.* Mongersen, an Oral SMAD7 Antisense Oligonucleotide, and Crohn's Disease. *N. Engl. J. Med.* **372**, 1104–1113 (2015).
60. ClinicalTrials.gov. Efficacy and Safety Study of Mongersen (GED-0301) for the Treatment of Subjects With Active Crohn's Disease.
61. Collison, L. W. *et al.* The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* **450**, 566–569 (2007).
62. Collison, L. W. *et al.* IL-35-mediated induction of a potent regulatory T cell population. *Nat. Immunol.* **11**, 1093–1101 (2010).
63. Wirtz, S., Billmeier, U., Mchedlidze, T., Blumberg, R. S. & Neurath, M. F. Interleukin-35 mediates mucosal immune responses that protect against T-cell-dependent colitis. *Gastroenterology* **141**, 1875–86 (2011).
64. Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. Regulatory T Cells and Immune Tolerance. *Cell* **133**, 775–787 (2008).
65. Chinen, T. *et al.* An essential role for the IL-2 receptor in Treg cell function. *Nat. Immunol.* **17**, 1322–1333 (2016).
66. Abbas, A. K., Trotta, E., R Simeonov, D., Marson, A. & Bluestone, J. A. Revisiting IL-2: Biology and therapeutic prospects. *Sci. Immunol.* **3**, (2018).
67. Fan, M. Y. *et al.* Differential Roles of IL-2 Signaling in Developing versus Mature Tregs. *Cell Rep.* **25**, 1204–1213.e4 (2018).
68. Waldmann, T. A. The Multi-Subunit Interleukin-2 Receptor. *Annu. Rev. Biochem.* **58**, 875–905 (1989).
69. de la Rosa, M., Rutz, S., Dorninger, H. & Scheffold, A. Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. *Eur. J. Immunol.* **34**, 2480–2488 (2004).
70. Pandiyan, P., Zheng, L., Ishihara, S., Reed, J. & Lenardo, M. J. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat. Immunol.* **8**, 1353–1362 (2007).
71. Sharfe, N., Dadi, H. K., Shahar, M. & Roifman, C. M. Human immune disorder arising from mutation of the  $\alpha$  chain of the interleukin-2 receptor. *Proc. Natl. Acad. Sci.* **94**, 3168–3171 (1997).
72. Caudy, A. A., Reddy, S. T., Chatila, T., Atkinson, J. P. & Verbsky, J. W. CD25 deficiency causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like syndrome, and defective IL-10 expression from CD4 lymphocytes. *J. Allergy Clin. Immunol.* **119**, 482–7 (2007).
73. Goudy, K. *et al.* Human IL2RA null mutation mediates immunodeficiency with lymphoproliferation and autoimmunity. *Clin. Immunol.* **146**, 248–261 (2013).
74. Caudy, A. A., Reddy, S. T., Chatila, T., Atkinson, J. P. & Verbsky, J. W. CD25 deficiency causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like syndrome, and defective IL-10 expression from CD4 lymphocytes. *J. Allergy Clin. Immunol.* **119**, 482–7 (2007).
75. Deaglio, S. *et al.* Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J. Exp. Med.* **204**, 1257–65 (2007).
76. Zarek, P. E. *et al.* A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells. *Blood* **111**, 251–259 (2008).
77. Kurtz, C. C. *et al.* Extracellular adenosine regulates colitis through effects on lymphoid and nonlymphoid cells. *Am. J. Physiol. Liver Physiol.* **307**, G338–G346 (2014).
78. Takahashi, T. *et al.* Immunologic self-tolerance maintained by CD25(+)CD4(+)



- regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med.* **192**, 303–10 (2000).
79. Qureshi, O. S. *et al.* Trans-endocytosis of CD80 and CD86: a molecular basis for the cell extrinsic function of CTLA-4. *Science* (80-. ). **332**, 600 (2011).
  80. Oderup, C., Cederbom, L., Makowska, A., Cilio, C. M. & Ivars, F. Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. *Immunology* **118**, 240–249 (2006).
  81. Wing, K. *et al.* CTLA-4 control over Foxp3+ regulatory T cell function. *Science* **322**, 271–5 (2008).
  82. Kuehn, H. S. *et al.* Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. *Science* (80-. ). **345**, 1623–1627 (2014).
  83. Lo, B. *et al.* Patients with LRBA deficiency show CTLA4 loss and immune dysregulation responsive to abatacept therapy. *Science* (80-. ). **349**, 436–40 (2015).
  84. Lopez-Herrera, G. *et al.* Deleterious mutations in LRBA are associated with a syndrome of immune deficiency and autoimmunity. *Am. J. Hum. Genet.* **90**, 986–1001 (2012).
  85. Gamez-Diaz, L. G. *et al.* The extended phenotype of LPS-responsive beige-like anchor protein (LRBA) deficiency. *J. Allergy Clin. Immunol.* **137**, 223–230 (2016).
  86. Yu, X. *et al.* The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat. Immunol.* **10**, 48–57 (2009).
  87. Joller, N. *et al.* Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. *Immunity* **40**, 569–81 (2014).
  88. Grossman, W. J. *et al.* Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood* **104**, 2840–2848 (2004).
  89. Ren, X. *et al.* Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4+CD25+ regulatory T cells. *Cell Death Differ.* **14**, 2076–2084 (2007).
  90. Garin, M. I. *et al.* Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. *Blood* **109**, 2058–2065 (2007).
  91. Arpaia, N. *et al.* A Distinct Function of Regulatory T Cells in Tissue Protection. *Cell* **162**, 1078–89 (2015).
  92. Beltrán, C. J. *et al.* Characterization of the novel ST2/IL-33 system in patients with inflammatory bowel disease. *Inflamm. Bowel Dis.* **16**, 1097–1107 (2010).
  93. Kobori, A. *et al.* Interleukin-33 expression is specifically enhanced in inflamed mucosa of ulcerative colitis. *J. Gastroenterol.* **45**, 999–1007 (2010).
  94. Schiering, C. *et al.* The alarmin IL-33 promotes regulatory T-cell function in the intestine. *Nature* **513**, 564–568 (2014).
  95. Esensten, J. H., Muller, Y. D., Bluestone, J. A. & Tang, Q. Regulatory T-cell therapy for autoimmune and autoinflammatory diseases: The next frontier. *J. Allergy Clin. Immunol.* **142**, 1710–1718 (2018).
  96. Sagoo, P., Lombardi, G. & Lechler, R. I. Regulatory T cells as therapeutic cells. doi:10.1097/MOT.0b013e328317a476
  97. Apostolou, I. & Von Boehmer, H. In vivo instruction of suppressor commitment in naive T cells. *J. Exp. Med.* **199**, 1401–1408 (2004).
  98. Hoffmann, P., Eder, R., Kunz-Schughart, L. A., Andreesen, R. & Edinger, M. Large-scale in vitro expansion of polyclonal human CD4 CD25 high regulatory T cells. (2004).

- doi:10.1182/blood-2004-01-0086
99. Gravano, D. M. & Vignali, D. A. A. The battle against immunopathology: infectious tolerance mediated by regulatory T cells. *Cell. Mol. Life Sci.* **69**, 1997–2008 (2012).
  100. Canavan, J. B. *et al.* Developing in vitro expanded CD45RA+regulatory T cells as an adoptive cell therapy for Crohn's disease. *Gut* **65**, 584–594 (2016).
  101. Bluestone, J. A. *et al.* Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Sci. Transl. Med.* **7**, 315ra189 (2015).
  102. Trzonkowski, P., Szaryńska, M., Myśliwska, J. & Myśliwski, A. Ex vivo expansion of CD4<sup>+</sup> CD25<sup>+</sup> T regulatory cells for immunosuppressive therapy. *Cytom. Part A* **75A**, 175–188 (2009).
  103. Golovina, T. N. *et al.* Retinoic Acid and Rapamycin Differentially Affect and Synergistically Promote the Ex Vivo Expansion of Natural Human T Regulatory Cells. *PLoS One* **6**, e15868 (2011).
  104. Putnam, A. L. *et al.* Clinical Grade Manufacturing of Human Alloantigen-Reactive Regulatory T Cells for Use in Transplantation. *Am. J. Transplant.* **13**, 3010–3020 (2013).
  105. Tresoldi, E. *et al.* Stability of human rapamycin-expanded CD4+CD25+ T regulatory cells. *Haematologica* **96**, 1357–1365 (2011).
  106. Trzonkowski, P. *et al.* Differences in Kinetics of Donor Lymphoid Cells in Response to G-CSF Administration May Affect the Incidence and Severity of Acute GvHD in Respective HLA-Identical Sibling Recipients. *Med. Oncol.* **21**, 81–94 (2004).
  107. Kennedy-Nasser, A. A. *et al.* Ultra low-dose IL-2 for GVHD prophylaxis after allogeneic hematopoietic stem cell transplantation mediates expansion of regulatory T cells without diminishing antiviral and antileukemic activity. *Clin. Cancer Res.* **20**, 2215–25 (2014).
  108. Koreth, J. *et al.* Interleukin-2 and Regulatory T Cells in Graft-versus-Host Disease. *N. Engl. J. Med.* **365**, 2055–2066 (2011).
  109. Taylor, P. A. *et al.* The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* **99**, 3493–9 (2002).
  110. Di Ianni, M. *et al.* Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* **117**, 3921–8 (2011).
  111. Trzonkowski, P. *et al.* First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127<sup>−</sup> T regulatory cells. *Clin. Immunol.* **133**, 22–26 (2009).
  112. Martelli, M. F. *et al.* HLA-haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents acute leukemia relapse. *Blood* **124**, 638–644 (2014).
  113. Gliwiński, M., Iwaszkiewicz-Grześ, D. & Trzonkowski, P. Cell-Based Therapies with T Regulatory Cells. *BioDrugs* **31**, 335–347 (2017).
  114. Mathew, J. M. *et al.* A Phase I Clinical Trial with Ex Vivo Expanded Recipient Regulatory T cells in Living Donor Kidney Transplants. *Sci. Rep.* **8**, 7428 (2018).
  115. Xia, G., He, J., Zhang, Z. & Leventhal, J. R. Targeting Acute Allograft Rejection by Immunotherapy With Ex Vivo-Expanded Natural CD4+CD25+ Regulatory T Cells. *Transplantation* **82**, 1749–1755 (2006).
  116. Xia, G., He, J. & Leventhal, J. R. Ex Vivo-Expanded Natural CD4+CD25+ Regulatory T Cells Synergize With Host T-Cell Depletion to Promote Long-Term Survival of



- Allografts. *Am. J. Transplant.* **8**, 298–306 (2008).
117. Joffre, O. *et al.* Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes. *Nat. Med.* **14**, 88–92 (2008).
  118. Tosello, V. *et al.* Differential expression of CCR7 defines two distinct subsets of human memory CD4+CD25+ Tregs. *Clin. Immunol.* **126**, 291–302 (2008).
  119. Gallon, L. *et al.* Differential Effects of Calcineurin and Mammalian Target of Rapamycin Inhibitors on Alloreactive Th1, Th17, and Regulatory T Cells. *Transplantation* **99**, 1774–1784 (2015).
  120. Chandran, S. *et al.* Polyclonal Regulatory T Cell Therapy for Control of Inflammation in Kidney Transplants. *Am. J. Transplant* **17**, 2945–2954 (2017).
  121. Safinia, N. *et al.* Successful expansion of functional and stable regulatory T cells for immunotherapy in liver transplantation. *Oncotarget* **7**, 7563–77 (2016).
  122. Li, W. *et al.* The Role of Foxp3+ Regulatory T Cells in Liver Transplant Tolerance. *Transplant. Proc.* **38**, 3205–3206 (2006).
  123. Li, Y. *et al.* Analyses of Peripheral Blood Mononuclear Cells in Operational Tolerance After Pediatric Living Donor Liver Transplantation. *Am. J. Transplant.* **4**, 2118–2125 (2004).
  124. Marek-Trzonkowska, N. *et al.* Administration of CD4+CD25highCD127- Regulatory T Cells Preserves  $\beta$ -Cell Function in Type 1 Diabetes in Children. *Diabetes Care* **35**, 1817–1820 (2012).
  125. Barzaghi, F. *et al.* Long-term follow-up of IPEX syndrome patients after different therapeutic strategies: An international multicenter retrospective study. *J. Allergy Clin. Immunol.* **141**, 1036–1049.e5 (2018).
  126. Holohan, D. R., Van Gool, F. & Bluestone, J. A. Thymically-derived Foxp3+ regulatory T cells are the primary regulators of type 1 diabetes in the non-obese diabetic mouse model. *PLoS One* **14**, e0217728 (2019).
  127. Fouser, G. *et al.* Following the fate of one insulin-reactive CD4 T cell: Conversion into Tregs and Tregs in the periphery controls diabetes in NOD mice. *Diabetes* **61**, 1169–1179 (2012).
  128. Chatenoud, L., Primo, J. & Bach, J. F. CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice. *J. Immunol.* **158**, 2947–54 (1997).
  129. Belghith, M. *et al.* TGF- $\beta$ -dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat. Med.* **9**, 1202–8 (2003).
  130. Herold, K. C. *et al.* Activation of human T cells by FcR nonbinding anti-CD3 mAb, hOKT3gamma1(Ala-Ala). *J. Clin. Invest.* **111**, 409–18 (2003).
  131. Keymeulen, B. *et al.* Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. *N. Engl. J. Med.* **352**, 2598–2608 (2005).
  132. Agace, W. W. Tissue-tropic effector T cells: generation and targeting opportunities. *Nat. Rev. Immunol.* **6**, 682–92 (2006).
  133. Hamann, A., Andrew, D. P., Jablonski-Westrich, D., Holzmann, B. & Butcher, E. C. Role of  $\alpha$ 4-integrins in lymphocyte homing to mucosal tissues in vivo. *J. Immunol.* **152**, 3282–93 (1994).
  134. Izcue, A. *et al.* Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. *Immunity* **28**, 559–70 (2008).
  135. Coombes, J. L., Robinson, N. J., Maloy, K. J., Uhlig, H. H. & Powrie, F. Regulatory T cells and intestinal homeostasis. *Immunol. Rev.* **204**, 184–194 (2005).

136. Ogino, H. *et al.* Regulatory T cells expanded by rapamycin in vitro suppress colitis in an experimental mouse model. *J. Gastroenterol.* **47**, 366–76 (2012).
137. Nishio, J. *et al.* Requirement of full TCR repertoire for regulatory T cells to maintain intestinal homeostasis. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 12770–5 (2015).
138. Scotta, C. *et al.* Differential effects of rapamycin and retinoic acid on expansion, stability and suppressive qualities of human CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T regulatory cell subpopulations. *Haematologica* **98**, 1291–1299 (2013).
139. Doorenspleet, M. E. *et al.* Profoundly Expanded T-cell Clones in the Inflamed and Uninflamed Intestine of Patients With Crohn's Disease. *J. Crohn's Colitis* **11**, 831–839 (2017).
140. Gulwani-Akolkar, B. *et al.* Selective expansion of specific T cell receptors in the inflamed colon of Crohn's disease. *J. Clin. Invest.* **98**, 1344–54 (1996).
141. Wu, J. *et al.* Expanded TCR $\beta$  CDR3 clonotypes distinguish Crohn's disease and ulcerative colitis patients. *Mucosal Immunol.* **11**, 1487–1495 (2018).
142. Wu, J. *et al.* Expanded TCR $\beta$  CDR3 clonotypes distinguish Crohn's disease and ulcerative colitis patients. *Mucosal Immunol.* **11**, 1487–1495 (2018).
143. Harbige, J., Eichmann, M. & Peakman, M. New insights into non-conventional epitopes as T cell targets: The missing link for breaking immune tolerance in autoimmune disease? *J. Autoimmun.* **84**, 12–20 (2017).
144. Rossig, C. CAR T cell immunotherapy in hematology and beyond. *Clin. Immunol.* **186**, 54–58 (2018).
145. Voskens, C. J. *et al.* Characterization and Expansion of Autologous GMP-ready Regulatory T Cells for TREG-based Cell Therapy in Patients with Ulcerative Colitis. *Inflamm. Bowel Dis.* **23**, 1348–1359 (2017).
146. Desreumaux, P. *et al.* Safety and Efficacy of Antigen-Specific Regulatory T-Cell Therapy for Patients With Refractory Crohn's Disease. *Gastroenterology* **143**, 1207–1217.e2 (2012).
147. Grainge, M. J., West, J. & Card, T. R. Venous thromboembolism during active disease and remission in inflammatory bowel disease: a cohort study. *Lancet* **375**, 657–663 (2010).
148. Xu, Y. *et al.* In Vivo Generation of Gut-Homing Regulatory T Cells for the Suppression of Colitis. *J. Immunol.* **202**, 3447–3457 (2019).
149. Scalapino, K. J., Tang, Q., Bluestone, J. A., Bonyhadi, M. L. & Daikh, D. I. Suppression of disease in New Zealand Black/New Zealand White lupus-prone mice by adoptive transfer of ex vivo expanded regulatory T cells. *J. Immunol.* **177**, 1451–9 (2006).
150. Scalapino, K. J. & Daikh, D. I. Suppression of Glomerulonephritis in NZB/NZW Lupus Prone Mice by Adoptive Transfer of Ex Vivo Expanded Regulatory T Cells. *PLoS One* **4**, e6031 (2009).
151. He, J. *et al.* Low-dose interleukin-2 treatment selectively modulates CD4<sup>+</sup> T cell subsets in patients with systemic lupus erythematosus. *Nat. Med.* **22**, 991–993 (2016).
152. Dall'Era, M. *et al.* Adoptive Regulatory T Cell Therapy in a Patient with Systemic Lupus Erythematosus. *Arthritis Rheumatol.* (2018). doi:10.1002/art.40737
153. Sugiyama, H. *et al.* CD4<sup>+</sup>CD25<sup>high</sup> regulatory T cells are markedly decreased in blood of patients with pemphigus vulgaris. *Dermatology* **214**, 210–20 (2007).
154. Taubert, R. *et al.* Intrahepatic regulatory T cells in autoimmune hepatitis are associated with treatment response and depleted with current therapies. *J. Hepatol.*

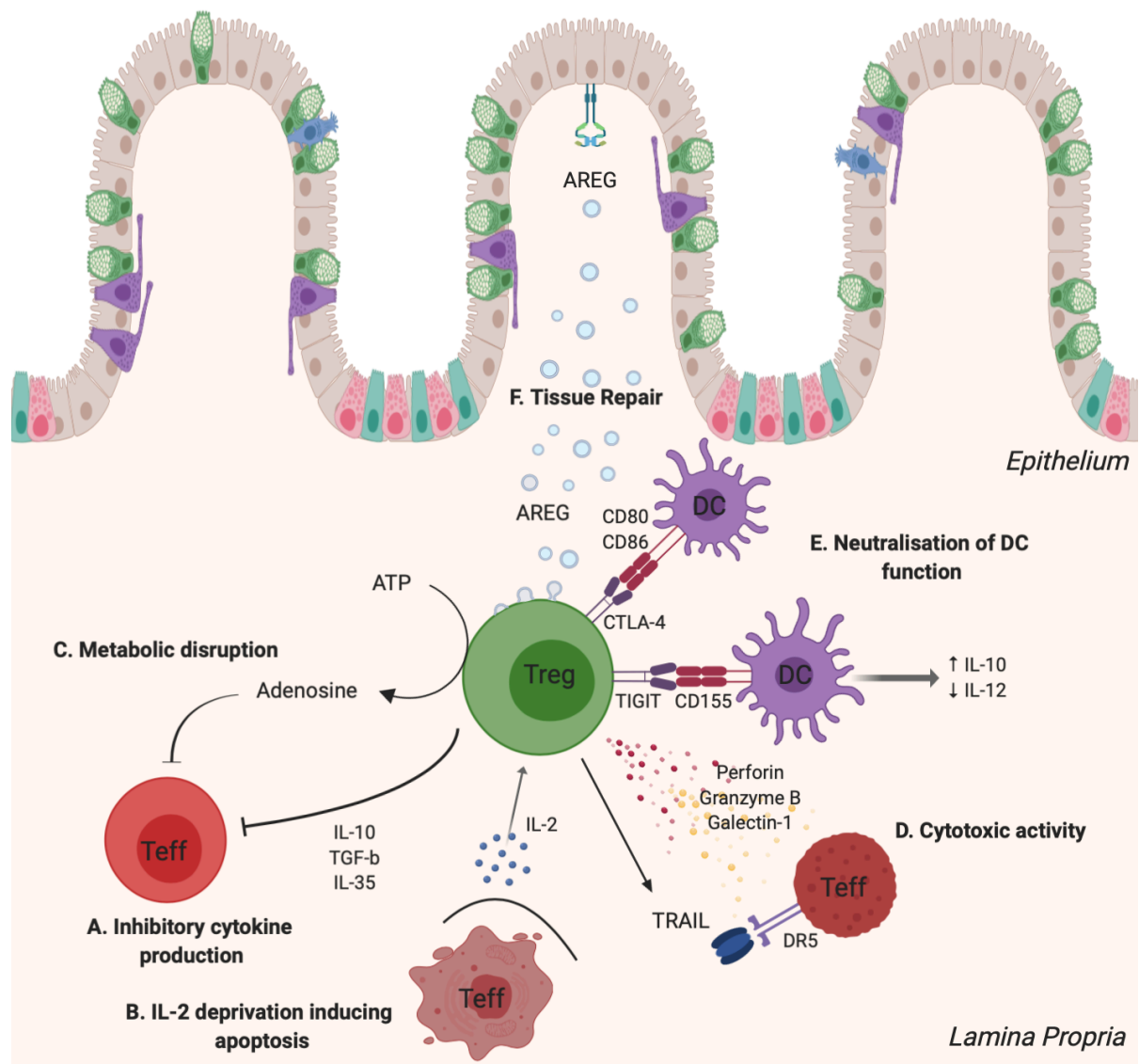
1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

**61**, 1106–1114 (2014).

155. Lee, J.-H. *et al.* The levels of CD4+CD25+ regulatory T cells in paediatric patients with allergic rhinitis and bronchial asthma. *Clin. Exp. Immunol.* **148**, 53–63 (2007).

156. Hartl, D. *et al.* Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. *J. Allergy Clin. Immunol.* **119**, 1258–1266 (2007).

157. Theil, A. *et al.* T cell receptor repertoires after adoptive transfer of expanded allogeneic regulatory T cells. *Clin. Exp. Immunol.* **187**, 316–324 (2017).



**Figure 1. Mechanisms of Treg mediated suppression**

Tregs utilize a multitude of mechanisms to promote a tolerogenic microenvironment and tissue repair. (A) Secretion of the anti-inflammatory cytokines, IL-10, TGF- $\beta$  and IL-35, not only inhibit Teff proliferation, but also suppress Th1 and Th17 effector function, both of which are key mediators of IBD. (B) Tregs express the high affinity IL-2 receptor  $\alpha$ -chain (CD25) consuming local IL-2 with greater affinity than effector cells. Teffs which are 'starved' of IL-2 exhibit restricted proliferation and undergo apoptosis. (C) Tregs co-expressing CD39 and CD73 disrupt metabolic processes in effector cells by converting ATP into peri-cellular adenosine, a potent inhibitor of Teff function. Additionally, adenosine stimulates TGF- $\beta$  production, promoting development of pTregs. (D) Tregs are capable of secreting perforin, granzyme B and galectin-1 which are directly cytotoxic against Teffs. Activated Tregs also express TRAIL, inducing apoptosis of Teffs through the TRAIL/DR5 pathway. (E) Expression of

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

CTLA-4 degrades DC-derived CD80 and CD86 leading to impaired CD28-mediated co-stimulation of T cells. DC function is further inhibited through the interaction of Treg-derived TIGIT and CD155 on DCs. This induces IL-10 production and suppresses IL-12. (F) In response to alarmins, Tregs produce AREG, an important regulator of tissue repair and regeneration. AREG, amphiregulin; ATP, adenosine tri-phosphate; CTLA-4, cytotoxic T lymphocyte associated protein 4; DC, dendritic cell; DR5, death receptor 5; IBD, inflammatory bowel disease; pTregs, peripheral regulatory T cells; Teff, T effector lymphocyte; TGF- $\beta$ , transforming growth factor beta; Th1, T helper 1 cell; Th17, T helper 17 cell; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; TRAIL, TNF-related apoptosis-inducing ligand; Treg, regulatory T cell. Figure generated using BioRender© illustration software.

# Regulatory T cell therapy in Crohn's disease: challenges and advances

Jennie N Clough,<sup>\*,1,2</sup> Omer S Omer,<sup>\*,1</sup> Scott Tasker,<sup>1</sup> Graham M Lord,<sup>1,3,#</sup> and Peter M Irving<sup>1,4,#</sup>

1. School of Immunology and Microbial Sciences, King's College London, UK
2. NIHR Biomedical Research Centre at Guy's and St Thomas' NHS Foundation Trust and King's College London, UK
3. Faculty of Biology Medicine and Health, University of Manchester, UK
4. Department of Gastroenterology, Guy's and St Thomas' NHS Foundation Trust

## \*Equal contribution

# Correspondence: [graham.lord@manchester.ac.uk](mailto:graham.lord@manchester.ac.uk) or [peter.irving@kcl.ac.uk](mailto:peter.irving@kcl.ac.uk) (020 7188 7188 x. 82497). LORD Lab, MRC Centre for Transplantation, King's College London, 5<sup>th</sup> Floor Tower Wing, Guy's Hospital, London, SE1 9RT.

Word Count: 5981

## ABBREVIATIONS:

- APC – antigen presenting cell  
AREG – amphiregulin  
ATP – adenosine triphosphate  
CD – Crohn's disease  
CDAI – Crohn's disease activity index  
CTLA-4 – cytotoxic T lymphocyte antigen 4  
DC – dendritic cell  
DR5 – death receptor 5  
DSS – dextran sulfate sodium  
Ebi3 – Epstein Barr virus induced 3  
EGF – epidermal growth factor  
FACS – fluorescence-activated cell sorting

1		
2		
3	33	Fgl2 – fibrinogen-like protein 2
4		
5	34	Foxp3 – Forkhead box P-3
6		
7	35	GALT – gut-associated lymphoid tissue
8		
9	36	GC-MS – gas chromatography mass spectrometry
10		
11	37	GI – gastrointestinal
12		
13	38	GMP – good manufacturing practice
14		
15	39	GvHD – graft versus host disease
16		
17	40	HEPA – high efficiency particulate air
18		
19	41	HSCT – haematopoietic stem cell transplant
20		
21	42	IBD – inflammatory bowel disease
22		
23	43	IFN $\gamma$ – interferon $\gamma$
24		
25	44	IL – interleukin
26		
27	45	IPEX – ‘immune dysregulation, polyendocrinopathy, enteropathy, X-linked’
28		
29	46	LPMC – lamina propria mononuclear cell
30		
31	47	MACS – magnetic bead-activated cell sorting
32		
33	48	MAdCAM-1 – mucosal vascular addressin cell adhesion molecule 1
34		
35	49	MHC – major histocompatibility complex
36		
37	50	MMP – matrix metalloproteinase
38		
39	51	NK – natural killer
40		
41	52	NOD – non-obese diabetic
42		
43	53	PBMC – peripheral blood mononuclear cell
44		
45	54	RAR $\alpha$ – retinoic acid receptor $\alpha$
46		
47	55	RORC – related orphan receptor C
48		
49	56	RPMI – Roswell Park Memorial Institute
50		
51	57	SCID – severe combined immunodeficiency
52		
53	58	SLE – systemic lupus erythematosus
54		
55	59	STAT – signal transducer and activator of transcription
56		
57	60	T1DM – type 1 diabetes mellitus
58		
59	61	TCR – T cell receptor
60		
	62	Teff – effector T cell
	63	TGF- $\beta$ – transforming growth factor $\beta$
	64	Th1 – T helper 1 cell



Th17 – T helper 17 cell  
TIGIT – T cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domains  
TNBS – 2,4,6-trinitrobenzene sulfonic acid  
TNF $\alpha$  – tumour necrosis factor  $\alpha$   
TRAIL – tumour necrosis factor-related apoptosis inducing ligand  
Treg – regulatory T cell  
pTreg – peripheral regulatory T cell  
tTreg – thymic regulatory T cell  
UC – ulcerative colitis

## ABSTRACT:

The prevalence of inflammatory bowel disease is rising in the Western world. Despite an increasing repertoire of therapeutic targets, a significant proportion of patients suffer chronic morbidity. Studies in mice and humans have highlighted the critical role of regulatory T cells in immune homeostasis, with defects in number and suppressive function of regulatory T cells seen in Crohn's disease patients. We review the function of regulatory T cells and the pathways by which they exert immune tolerance in the intestinal mucosa. We explore the principles and challenges of manufacturing a cell therapy, and discuss clinical trial evidence to date for their safety and efficacy in human disease, with particular focus on the development of a regulatory T cell therapy for Crohn's disease.

**Keywords:** Crohn's disease, Immunology, Immunoregulation, Intestinal T cell, T lymphocytes

## INTRODUCTION:

Inflammatory bowel disease (IBD), chiefly comprising Crohn's disease (CD) and ulcerative colitis (UC), is a chronic inflammatory group of disorders of the gastrointestinal (GI) tract arising from overexuberant innate and adaptive immune responses to environmental factors in genetically susceptible individuals. IBD affects at least 0.5% of the population in the Western world with 1 million sufferers in USA and 2.5 million in Europe.<sup>1</sup> Global



prevalence continues to increase, largely driven by rising numbers of patients in newly industrialised regions including India and Asia.<sup>1</sup> The burden of disease is significant with 20-25% of patients experiencing chronic continuous symptoms which contributes to higher rates of unemployment, sick leave and permanent work disability.<sup>2</sup> Even with an aggressive top-down approach to therapy, the majority of patients fail to achieve prolonged, steroid-free remission and are at particular risk of requiring surgical intervention. Cumulative surgery rates in CD are high in Europe with 30-50% of patients requiring surgical intervention and up to 20% needing a reoperation 5-10 years from diagnosis.<sup>2</sup>

As our understanding of the pathophysiology of IBD and its socioeconomic impact has evolved, there has been great impetus to identify novel therapeutic targets to add to the existing arsenal of immunomodulators and biologics. These have focussed on a variety of areas including targeting lymphocyte trafficking (vedolizumab, ozanimod, anti-MAdCAM1) and activation (anti-IL6, anti-IL12/IL23), modulating intestinal barrier function (phosphatidylcholine), matrix remodelling (STNM-01, MMP9 blocker) and manipulation of gut microbiota (faecal microbiota transplant).<sup>3</sup> An important pathological process increasingly recognised as driving intestinal inflammation and autoimmunity is the loss of immune homeostasis secondary to qualitative or quantitative defects in the regulatory T cell (Treg) pool.

Tregs are CD4<sup>+</sup> T cells that characteristically express the high affinity IL-2 receptor  $\alpha$ -chain (CD25), and master transcription factor Forkhead box P-3 (Foxp3), which is essential for their suppressive phenotype and stability.<sup>4-6</sup> As activated CD4<sup>+</sup> T cells can upregulate CD25 expression, an additional defining feature of Tregs is the absence of IL-7 receptor  $\alpha$ -chain (CD127).<sup>7</sup> Their primary function is as dominant controllers of self-tolerance, tissue inflammation and long-term immune homeostasis. Despite making up only 5-10% of the peripheral CD4<sup>+</sup> T cell pool, Tregs exert powerful inhibitory effects on effector cells through a variety of mechanisms including cytokine secretion, metabolic disruption, inhibition of dendritic cells (DCs) and cytotoxicity. These mechanisms have been rigorously examined using animal models and shown to protect against the development of intestinal inflammation. Studies in patients with IBD have identified defects in the number and distribution of Tregs, and their ability to traffic to the GI tract.<sup>8</sup> Additionally, resistance to Treg-mediated

1  
2  
3 129 suppression has been noted in lamina propria T effector cells (Teffs).<sup>9</sup> These factors are  
4  
5 130 likely to be pivotal in driving intestinal inflammation.  
6

7 131

8  
9 132 There is growing interest in the therapeutic potential of adoptively transferring healthy  
10  
11 133 Tregs into patients with a wide range of conditions, including IBD and autoimmune disease,  
12  
13 134 in an attempt to shift the balance in areas of active inflammation towards a more  
14  
15 135 tolerogenic microenvironment. Early phase clinical trials have already reported in the fields  
16  
17 136 of solid organ transplantation, graft-versus-host disease (GvHD) and type 1 diabetes mellitus  
18  
19 137 (T1DM) with reassuring safety data and potential signals of efficacy.  
20

21 138

22 139 This review provides a summary of the suppressive mechanisms utilised by Tregs and  
23  
24 140 highlights seminal work linking intestinal inflammation with loss of Treg function in both  
25  
26 141 animal models of disease and in humans. Additionally, we review ongoing clinical trials with  
27  
28 142 Treg therapy and outline an entirely novel therapeutic strategy for CD using Tregs expanded  
29  
30 143 under GMP (Good Manufacturing Practice) conditions that will be adoptively transferred to  
31  
32 144 patients in an attempt to ameliorate intestinal inflammation and restore immune  
33  
34 145 homeostasis.  
35

36 146

#### 37 147 **TREGS IN HEALTH AND DISEASE:**

38 148

39  
40 149 Tregs can be broadly divided into two groups, thymic Tregs (tTregs) or peripherally induced  
41  
42 150 Tregs (pTregs) based on their developmental origin. Tregs generated in the thymus (tTregs)  
43  
44 151 in the early neonatal period migrate to peripheral organs where they maintain tolerance.  
45  
46 152 This was discovered in 1969 by Nishizuka and Sakakura who showed that in mice,  
47  
48 153 thymectomy 3 days after birth led to the depletion of Foxp3<sup>+</sup> Tregs and development of  
49  
50 154 autoimmune oophoritis.<sup>10</sup> In contrast, mice who had thymectomy at day 7 remained healthy  
51  
52 155 as the tTregs had already migrated to the periphery by this point.<sup>11</sup> Over a decade later,  
53  
54 156 Sakaguchi *et al* demonstrated that day-3 thymectomy autoimmune oophoritis could be  
55  
56 157 prevented with CD4<sup>+</sup> T cell inoculation from healthy syngeneic donors. Conversely, the  
57  
58 158 adoptive transfer of T cells from these sick mice were capable of inducing autoimmune  
59  
60 159 disease in healthy T cell deficient mice.<sup>12</sup> Similar findings were noted in rats that underwent  
160  
160 160 adult thymectomy and irradiation resulting in lymphopenia, autoimmune diabetes and

insulinitis. An injection of CD45RC(low) T cells from healthy donors were capable of preventing disease.<sup>13</sup> Mottet *et al* subsequently described CD25-expressing CD4<sup>+</sup> T cells that were able to cure established T cell transfer colitis.<sup>14</sup> By the early 2000's it was clear that a thymically-derived CD4<sup>+</sup>CD25<sup>+</sup> T cell population possessed the ability to suppress autoreactive T cells and eliminate autoimmunity.

Peripherally induced Tregs (pTregs) were first described in 2003 where naïve CD4<sup>+</sup>CD25<sup>-</sup> T cells could be converted into Foxp3-expressing CD4<sup>+</sup>CD25<sup>+</sup> Tregs by T cell receptor (TCR) co-stimulation in the presence of TGF- $\beta$ .<sup>15</sup> pTreg conversion in gut-associated lymphoid tissues (GALT) was enhanced when naïve CD4<sup>+</sup> T cells encountered antigen in the presence of TGF- $\beta$ , IL-2 and retinoic acid (RA).<sup>16,17</sup> This is facilitated by CD103<sup>+</sup> DCs conditioned by the intestinal microenvironment to produce or activate TGF- $\beta$  and provide RA.<sup>18,19</sup> In the absence of CD103 expression, DCs fail to induce Treg development and produce proinflammatory cytokines.<sup>18,20</sup> Additionally, in patients with UC, CD103<sup>+</sup> DCs appear to have impaired ability to generate pTregs, but induce colitogenic T helper (Th) 1, Th2 and Th17 responses suggesting CD103<sup>+</sup> DC-mediated pTreg induction is functionally relevant in IBD pathogenesis.<sup>21</sup>

Distinguishing tTregs from pTregs can be difficult as no definitive markers exist. Recently, the expression of the membrane protein neuropilin-1 (Nrp1) and the transcription factor Helios by tTregs but not by pTregs has been used to differentiate Treg subsets.<sup>22</sup> The significance of this lies in the epigenetic differences in the *Foxp3* locus rendering pTregs less stable and more likely to demonstrate plasticity towards a Th17 cell phenotype under inflammatory conditions.<sup>23</sup> The developmental origin of Tregs selected for expansion as a cell therapy product is therefore an important consideration and will be addressed in more detail later in this review.

The first study identifying Tregs in humans was published in 2001. Baecher-Allan *et al* characterised CD4<sup>+</sup>CD25<sup>+</sup> T cells in the thymus and peripheral blood which exhibited anti-inflammatory and suppressive properties.<sup>24</sup> Subsequent work established Foxp3 as the master transcription factor for Tregs.<sup>4,6,25</sup> Foxp3 can however be expressed transiently in non-regulatory CD4<sup>+</sup> T cells upon TCR activation and the CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> surface

phenotype must be used to define Tregs.<sup>26</sup> Inactivating mutations in *Foxp3* clinically manifest as severe autoimmunity with a scurfy phenotype in mice and IPEX syndrome ('immune dysregulation, polyendocrinopathy, enteropathy, X-linked') in humans.<sup>27–30</sup> With autoimmune enteropathy (manifesting as chronic diarrhoea and malabsorption) a predominant feature, attention was focussed on the functional role of Tregs within the GI tract.

Peripheral Tregs are found in abundance in the intestinal lamina propria where interactions with environmental antigens can shape phenotypic differences and transcription factor expression.<sup>31</sup> The gut microbiota represents a substantial antigen load driving the expansion of colonic pTregs that co-express the Th17 master transcription factor ROR $\gamma$ t.<sup>32</sup> These Foxp3<sup>+</sup> ROR $\gamma$ t<sup>+</sup> pTregs have a stable regulatory phenotype and provide tolerance against the gut microbiota.<sup>33,34</sup> Conversely, ROR $\gamma$ t<sup>+</sup> pTregs are found in the small intestine where they are induced by dietary antigens and repress underlying Th1 cell responses to ingested proteins.<sup>35</sup> Finally, an intestinal tTreg population that co-express the Th2 master transcription factor, GATA3, has been shown to mediate repair of the intestinal mucosa. GATA3<sup>+</sup> tTregs express high levels of the IL-33 receptor, ST2, and amphiregulin, an epidermal growth factor receptor ligand involved in tissue repair.<sup>36,37</sup>

Following on from the fundamental observations linking Treg dysfunction to an array of autoimmune polyendocrine syndromes, studies began to emerge identifying defects in either number or function of peripheral blood Tregs in autoimmune disorders including IBD, type 1 diabetes, multiple sclerosis, systemic lupus erythematosus (SLE), myasthenia gravis and rheumatoid arthritis.<sup>8,38–42</sup> Maul *et al* observed that in patients with active IBD, the intestinal lamina propria Treg pool was significantly smaller than that of a positive control, namely diverticulitis.<sup>8</sup> Additionally, in these patients, the peripheral blood Treg pool was smaller than that of inactive IBD or diverticulitis.<sup>8</sup> Interestingly, the peripheral blood Tregs retained their suppressive capacity suggesting that disease may be driven by ineffective trafficking to the gut and reduced numbers of Tregs. Furthermore, colitogenic T cells from IBD patients appear to be resistant to TGF- $\beta$ 1-mediated Treg suppression highlighting an additional defect in immunological tolerance that may drive disease.<sup>43</sup>

**TREG FUNCTION AND COLITIS:**

Tregs function as key mediators of peripheral tolerance through direct cellular contact and paracrine actions on tissues where they reside.<sup>44,45</sup> It is essential that Tregs effectively traffic to target organs where they promote a tolerogenic microenvironment. An important example is IL-10-secreting Tregs that reside in the GI mucosa and control inflammatory responses induced by environmental insults. Selective disruption of IL-10 expression in these Tregs has been shown to cause spontaneous colitis.<sup>46</sup> This is one of many modalities that Tregs can employ to maintain immune homeostasis at the mucosal interface. Others include inhibitory cytokine secretion, cytolysis of effector cells, metabolic disruption, neutralization of antigen presenting cells (APC) and promotion of tissue repair.<sup>47</sup> These functions will be reviewed in further detail outlining their associations with intestinal inflammation (see Figure 1).

**Inhibitory Cytokines:**

The Treg cytokine repertoire includes the anti-inflammatory molecules IL-10, TGF- $\beta$  and IL-35. The expression of IL-10 and IL-35 requires TCR signalling, suggesting that Treg function in part relies on antigen encounter in the local microenvironment.<sup>48</sup> Pioneering work by Powrie *et al* over 20 years ago showcased the potent inhibitory ability of IL-10, where recombinant IL-10 therapy ameliorated established T cell transfer colitis.<sup>49</sup> Subsequently, the co-transfer of CD45RB(low) T cells were shown to prevent colitis and IL-10 was identified as an essential mediator for this *in vivo* suppression.<sup>50</sup> The suppressive effects of Treg-derived IL-10 in mice appear to be specific for mucosal surfaces rather than controlling systemic autoimmunity.<sup>46</sup> Further studies have demonstrated that IL-10 induces robust activation of a STAT3-dependent Th17 suppression program in Tregs, downstream of IL-10R.<sup>51</sup> This suppresses pathogenic Th17 cell responses and ablation of IL-10R in Tregs has been shown to cause colitis. It is therefore plausible that disordered IL-10 signalling may contribute to aberrant Th17 activity, which is implicated in IBD.<sup>52</sup> In fact, there have been several cases of homozygous loss-of-function mutations in *IL-10* and *IL-10R* arising in individuals from consanguineous marriages. These resulted in infantile severe, progressive, intractable Crohn's-like colitis.<sup>53</sup>

257

TGF- $\beta$  plays an important role inducing pTreg formation upon antigen encounter in GALT and has a functional role in suppressing pro-inflammatory pathways.<sup>54</sup> Tregs are capable of producing TGF- $\beta$ , which profoundly suppresses the proliferation of Teffs.<sup>55</sup> Treg-derived TGF- $\beta$ 1 inhibits Th1-cell differentiation and IBD in a transfer model of colitis.<sup>56</sup> Conversely, Tregs from TGF- $\beta$ 1-deficient mice fail to suppress intestinal inflammation in a SCID transfer model of colitis.<sup>55</sup> Human studies have supported these early findings; a study on healthy human colonic biopsies and lamina propria mononuclear cells (LPMC) treated with anti-TGF- $\beta$  neutralising antibody showed that TGF- $\beta$  is a critical suppressor of T-bet-dependent Teff proliferation and Th1 cytokine expression.<sup>57</sup> This suggests a role for TGF- $\beta$  in suppressing intestinal inflammation in humans. Indeed, MacDonald *et al* have shown that colonic tissue and isolated T cells from patients with CD overexpress Smad7, an inhibitor of TGF- $\beta$ 1 signalling.<sup>58</sup> Furthermore, colonic LPMCs from CD patients were resistant to Treg-mediated suppression, a phenomenon that could be reversed with Smad7 antisense treatment.<sup>43</sup> Smad 7 antisense therapy (Mongersen) was subsequently evaluated in CD but, despite promising early phase data, a phase III clinical trial was terminated early due to lack of benefit.<sup>59,60</sup> Although Mongersen may overcome Teff resistance to TGF- $\beta$ , it is possible in CD there are insufficient numbers of functional Tregs in the mucosal environment to produce TGF- $\beta$  explaining the disappointing trial outcome .

276

IL-35 is a heterodimer of Ebi3 and IL-12 $\alpha$  that is constitutively expressed in Foxp3<sup>+</sup> Tregs but not Teffs. It was first described in 2007 where *Ebi3*<sup>-/-</sup> and *IL-12 $\alpha$* <sup>-/-</sup> Tregs were shown to have significantly reduced regulatory activity *in vitro* and failed to cure T cell transfer colitis *in vivo*.<sup>61</sup> Additionally, IL-35 can induce the generation of a regulatory population from naïve mouse or human CD4<sup>+</sup> T cells. These so-called iT(R)35 cells mediate suppression via IL-35 alone, do not express Foxp3, and are strongly suppressive and stable *in vivo*.<sup>62</sup> In both dextran sulphate sodium (DSS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis, recombinant IL-35 therapy can treat disease through downregulation of the Th1 and Th17 master transcription factors, T-bet and RORC, respectively, and through inhibition of IFN- $\gamma$ , IL-6 and IL-17.<sup>63</sup>

287

**Inhibition of Metabolic Processes:**

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

289

290 While Tregs are not known to produce IL-2, their development and function is critically

291 dependent on this cytokine. IL-2 and the transcription factor STAT5, downstream of IL-2

292 receptor (IL-2R), induce the expression of Foxp3 and differentiation of tTregs.<sup>64</sup>

293 Furthermore, STAT5 activation driven by IL2R signalling enhances the suppressor function of

294 differentiated Tregs.<sup>65</sup> An absence of IL-2 signalling has been shown to reduce the number

295 and functional activity of Tregs, predisposing to autoimmunity and inflammation.<sup>66,67</sup> The

296 structural conformation of IL-2R in Tregs provides a competitive advantage for IL-2-receptor

297 engagement over alternative cell subsets. Tregs abundantly express IL-2 receptor  $\alpha$ -chain

298 (CD25), which together with the common  $\gamma$ -chain ( $\gamma$ c, CD132) and IL-2 receptor  $\beta$ -chain

299 (CD122) form a characteristic three subunit receptor configuration. This confers a ~1000-

300 fold increase in receptor affinity for IL-2 over Teffs.<sup>68</sup> In a pro-inflammatory environment

301 dominated by actively dividing effector cells, Tregs have the ability to “consume” local IL-2,

302 starving effector cells of this essential cytokine for survival and proliferation.<sup>45,69</sup> Moreover,

303 this mechanism has been shown to induce the apoptosis of effector cells.<sup>70</sup> This highlights

304 an important TCR-independent paracrine mode of suppression in local tissues, facilitated

305 through the constitutive expression of high affinity IL-2R (containing CD25). There have

306 been a handful of cases of CD25 deficiency in humans often manifesting in an IPEX-like

307 syndrome.<sup>71–73</sup> A notable case who presented with autoimmune enteropathy at 6 months

308 had Foxp3<sup>+</sup> Tregs with defective IL-10 expression suggesting that IL-2 responsiveness is

309 important for Treg-mediated IL-10 production.<sup>74</sup>

310

311 Tregs can also interfere with adenosine triphosphate (ATP) metabolism to dampen pro-

312 inflammatory responses. Tregs co-express the ectoenzymes CD39 and CD73 responsible for

313 the degradation of ATP and generation of pericellular adenosine.<sup>75</sup> Adenosine stimulates the

314 A2A receptor on Teffs exerting potent inhibitory effects. Activation of the A2A receptor also

315 inhibits IL-6 expression while enhancing the production of TGF- $\beta$ .<sup>76</sup> This promotes the

316 development of adaptive induced Tregs and simultaneously inhibits pro-inflammatory Th17

317 cell formation. Furthermore, signalling through the A2A receptor appears to control *in vivo*

318 murine colitis.<sup>77</sup>

319

320 **Neutralisation of Dendritic Cell Function:**



321

322 The activation of T cells requires TCR-antigen/MHC engagement in the context of a  
323 secondary signal, namely T cell-derived CD28 binding the DC B7 ligands, CD80 and CD86.  
324 This process is negatively regulated through the production of cytotoxic T lymphocyte  
325 antigen 4 (CTLA-4) which is constitutively expressed in Foxp3<sup>+</sup> Tregs.<sup>78</sup> CTLA-4-expressing  
326 cells can capture CD80 and CD86 by a process of trans-endocytosis and degrade these  
327 ligands, resulting in impaired co-stimulation via CD28.<sup>79</sup> This is a functionally significant  
328 process with Treg-conditioned DCs inducing poor T cell proliferation.<sup>80</sup> An additional  
329 mechanism mediated through the interaction of CTLA-4 and CD80/CD86 is the upregulation  
330 of indoleamine 2, 3-deoxygenase in DCs. This is a potent regulatory molecule which  
331 catabolises the essential amino acid tryptophan to the pro-apoptotic metabolite kynurenine  
332 leading to suppression of Teff function.<sup>64</sup> *In vivo* models have demonstrated that CTLA-4 is  
333 essential in preventing autoimmunity. Selective deletion of CTLA-4 in Tregs of BALB/c mice  
334 results in fatal T cell mediated autoimmune disease at just 20 days of age.<sup>81</sup> Additionally,  
335 several cases of germline heterozygous mutations in CTLA-4 have been identified in  
336 humans.<sup>82</sup> CTLA-4 haploinsufficiency resulted in dysregulation of Tregs, hyperactivation of  
337 Teffs and lymphocytic infiltration of target organs including the GI tract. It was recently  
338 discovered that LRBA (lipopolysaccharide-responsive and beige-like anchor protein)  
339 regulates CTLA-4 expression, where mutations in LRBA lead to reduced levels of CTLA-4.<sup>83</sup>  
340 These mutations are commonly associated with primary immunodeficiency, reduced Treg  
341 numbers and susceptibility to IBD.<sup>84,85</sup>

342

343 Recently, the coinhibitory molecule TIGIT has been described as an inhibitor of autoimmune  
344 responses through its interactions with DCs and T cells. TIGIT interacts with its ligand CD155  
345 on DCs to induce IL-10 and suppress IL-12 production, thereby inhibiting Th1 responses.<sup>86</sup> As  
346 Tregs are the primary cell type that constitutively express TIGIT, it has been suggested that  
347 the observed effects on DCs are mediated by TIGIT<sup>+</sup> Tregs. Furthermore, Tregs expressing  
348 TIGIT have been shown to directly suppress Th1 and Th17 responses through the production  
349 of the effector molecule fibrinogen-like protein 2 (Fgl2).<sup>87</sup>

350

351 **Cytotoxic Activity:**

352

1  
2  
3 353 Historically, cytotoxic activity has been associated with natural killer (NK) cells and cytotoxic  
4 354 T lymphocytes (CD8<sup>+</sup> T cells). In 2004, Grossman *et al* first described granzyme-B expressing  
5 355 CD4<sup>+</sup> Tregs capable of killing target cells in a perforin-dependent, but TCR-independent  
6 356 manner.<sup>88</sup> Boissonnas *et al* subsequently showed that in a mouse tumour model, Foxp3<sup>+</sup> T  
7 357 cells can kill antigen-specific DCs. Treg cytotoxicity has also been observed against CD4<sup>+</sup> T  
8 358 cells in both *in vitro* and *in vivo* models. Activated Tregs upregulate tumour necrosis factor-  
9 359 related apoptosis inducing ligand (TRAIL) which enhances suppressive activity as well as  
10 360 cytotoxicity against CD4<sup>+</sup> T cells. This is entirely dependent on the TRAIL/death receptor 5  
11 361 (DR5) pathway.<sup>89</sup> Galectin-1, a  $\beta$ -galactoside-binding protein known to induce T cell  
12 362 apoptosis has also been implicated in Treg cytotoxic function. Galectin-1 was found to be  
13 363 overexpressed in Tregs and galectin-1 knockout models were shown to possess reduced  
14 364 regulatory activity.<sup>90</sup>

15 365  
16 366 **Tissue Repair:**  
17 367  
18 368 Aside from limiting mucosal damage through the suppression of pro-inflammatory cells  
19 369 following environmental insults like infection, Tregs may also promote tissue repair.  
20 370 Recently, the epidermal growth factor (EGF)-like molecule amphiregulin (AREG) has gained  
21 371 attention as an important regulator of tissue repair and regeneration. In a murine model of  
22 372 influenza, selective Treg deficiency in AREG leads to severe acute lung damage without any  
23 373 alterations in Treg suppressor function. This suggests that Tregs play a direct role in tissue  
24 374 repair and maintenance that is distinct from their suppressive function.<sup>91</sup> Treg production of  
25 375 AREG is dependent on IL-18 or IL-33 which function as endogenous danger signals or  
26 376 alarmins, in response to tissue damage.<sup>91</sup> Studies in humans have revealed high levels of IL-  
27 377 33 in inflamed lesions of IBD patients, and Tregs expressing the IL-33 receptor, ST2, are  
28 378 enriched in the colon.<sup>92-94</sup> IL-33-Treg signalling may therefore represent an important  
29 379 pathway in both disease pathogenesis and recovery.

30 380  
31 381 **TREGS AS A THERAPEUTIC PRODUCT:**  
32 382  
33 383 In light of the vast array of preclinical data showcasing how a multitude of defects in Treg  
34 384 function contribute to autoimmunity and inflammation, including IBD, there has been great

interest in harnessing the suppressive ability of Tregs as a therapeutic product. Consequently, there are over 50 registered trials of Treg therapy that are either completed or ongoing (clinicaltrials.gov). Most of these trials involve adoptive cell transfer, although the dose of Tregs given is highly variable. In the setting of autoimmune disease and transplantation, the goals of treatment are the restoration of peripheral self tolerance, the suppression of inflammation and promotion of tissue repair.<sup>95</sup>

In order to become a successful therapeutic product, Tregs must home to sites of inflammation and secondary lymphoid tissues, and must undergo TCR engagement. It has been demonstrated in solid organ transplantation that alloantigen-specific Tregs provide higher therapeutic benefits than polyclonal Tregs, without delivering a systemic immunosuppressive effect.<sup>96</sup> Directing Tregs against a specific alloantigen also permits immunomodulatory functions to be concentrated at the site of the alloantigen source, circumventing the relative paucity of Tregs. An early study demonstrated that peripheral Treg expansion in mice could be driven by prolonged low dose subcutaneous infusion of a specific peptide.<sup>97</sup> The induced Tregs had suppressive abilities, and demonstrated high levels of Foxp3 expression indicating a stable Treg phenotype. However, in IBD, a specific antigen has yet to be identified.

The relative paucity of Tregs in peripheral blood represents an obstacle to the development of a cellular therapy, though the optimum number of Tregs to be infused remains unclear. It has been suggested that the number of Tregs given should be at least as great as the number of T cells in the body,<sup>98</sup> though Tregs also exhibit the ability to confer suppressive ability on conventional T cells through 'infectious tolerance'.<sup>96</sup> In this process, the direct secretion of TGF- $\beta$ , IL-10 and IL-35 by Tregs, and indirect induction via DCs, can generate a regulatory microenvironment which may partially circumvent the problem of low absolute numbers of Tregs.<sup>99</sup>

Several groups have developed protocols in line with GMP requirements to permit *ex vivo* cell expansion of Tregs.<sup>98,100,101</sup> GMP-manufactured Tregs delivered in some early trials were only around 50% pure, but the development of plastic beads coated with stimulatory antibodies and the discovery of additional surface markers for Treg phenotyping mean that

1  
2  
3 417 a product with purity greater than 90% is now achievable.<sup>98</sup> Contamination of the expansion  
4  
5 418 product with Teffs hampers expansion,<sup>102</sup> but the inclusion of rapamycin in cell culture  
6  
7 419 blocks expansion of Teffs without affecting Treg proliferation, leading to the preferential  
8  
9 420 promotion of Treg proliferation.<sup>98,103</sup>

10 421  
11  
12 422 Tregs are first isolated from peripheral blood by surface marker expression  
13 423 (CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup>). This can be performed using stream in air fluorescence-activated cell  
14  
15 424 sorters (FACS) which yield a highly pure starting population, but the necessary air exposure  
16  
17 425 requires high efficiency particulate air (HEPA) enclosures, and single use sample lines to be  
18  
19 426 compatible with manufacturing GMP cell products. Closed system magnetic bead-activated  
20  
21 427 cell sorting (MACS) can be adapted for large-scale isolation of human Tregs, but unlike FACS  
22  
23 428 cannot easily distinguish surface marker expression density. A recently developed  
24  
25 429 microfluidic chip fluorescence-activated cell sorter, the MACSQuant Tyto (Miltenyi Biotech,  
26  
27 430 Germany) surmounts the problems of stream in air sorters, as the cells remain in a closed  
28  
29 431 system throughout the sorting process. Expansion of the sorted cells is achieved through  
30  
31 432 polyclonal TCR activation with anti-CD3/anti-CD28 beads.<sup>104</sup> Tregs are sampled and checked  
32  
33 433 for sterility and phenotype throughout the expansion process. With optimised conditions, a  
34  
35 434 500-fold expansion can be anticipated over a 14 day period.<sup>101</sup>

36 435  
37  
38 436 Uncertainty about the plasticity of Tregs in culture and following infusion means there is a  
39  
40 437 theoretical concern about the development of a pro-inflammatory phenotype, which could  
41  
42 438 lead to transplant rejection or aggravation of inflammation. However, rapamycin-expanded  
43  
44 439 Tregs are not contaminated by IL-17-producing Th17 cells, and these cells maintain a stable  
45  
46 440 phenotype on transfer *in vivo* to mice.<sup>105</sup> Canavan *et al.* found that the starting population  
47  
48 441 for Treg expansion from the peripheral blood of CD patients has a critical effect on the  
49  
50 442 phenotype of the expanded cell population.<sup>100</sup> Tregs from a highly pure FACS-sorted 'naïve'  
51  
52 443 CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup>CD45RA<sup>+</sup> precursor population demonstrated enhanced suppressive  
53  
54 444 ability and reduced Th17 plasticity *in vitro* compared to a FACS-sorted  
55  
56 445 CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup>CD45RA<sup>-</sup> or MACS-enriched CD8<sup>-</sup>CD25<sup>+</sup> population. Rapamycin appears to  
57  
58 446 imprint a fixed CD4<sup>+</sup>CD25<sup>hi</sup> phenotype to cells expanded from a 'naïve' CD45RA<sup>+</sup> population,  
59  
60 447 as evidenced by the retention of demethylation at the Foxp3 locus.

## TREG THERAPY IN OTHER CONDITIONS:

There is an increasing body of evidence for the use of Tregs as cellular therapy in autoimmune disease and transplantation (see Table 1). Adoptive transfer of Tregs to prevent GvHD was the first illustration of the potent therapeutic potential of Tregs in experimental transplantation.

Study	Clinical context	Enrichment protocol	Expansion protocol	Dose	Study outcome
Trzonkowski <i>et al.</i> (2009)	Treatment of acute and chronic GvHD N=2	Tregs from allogenic buffy coat. CD4 <sup>+</sup> negative bead selection followed by FACS-based sorting of CD4 <sup>+</sup> CD25 <sup>hi</sup> CD127 <sup>lo</sup> cells	RPMP 1640 with 10% autologous plasma IL-2 (1000IU/ml) Anti-CD3/anti-CD28 beads (1:1) 3 weeks	Acute GvHD: 1x10 <sup>6</sup> /kg Chronic GvHD: 3x10 <sup>6</sup> /kg	Transient improvement in acute GvHD; alleviation of symptoms and reduction of immunosuppression in chronic GvHD
Brunstein <i>et al.</i> (2011)	Prevention of GvHD following umbilical cord blood transplantation N=23	CD25 <sup>+</sup> bead positive selection	X-Vivo 15 with 10% human AB serum IL-2 (300IU/ml) Anti-CD3/anti-CD28 beads (1:2) 18±1 days	0.1-30x10 <sup>5</sup> /kg	Well –tolerated; reduced incidence of grade II-IV GvHD in Treg recipients
Marek-Trzonkowska <i>et al.</i> (2012)	Safety of autologous <i>in vitro</i> expanded Tregs in paediatric type 1 diabetes N=10	FACS-based sorting of CD3 <sup>+</sup> CD4 <sup>+</sup> CD25 <sup>hi</sup> CD127 <sup>lo</sup> cells	CellGro medium with 10% autologous plasma IL-2 (1000IU/ml) Anti-CD3/anti-CD28 beads (1:1) Up to 2 weeks	10-20x10 <sup>6</sup> /kg	Well-tolerated; decreased insulin requirements and C-peptide levels in Treg recipients
Desreumaux <i>et al.</i> (2012)	Safety and efficacy in Crohn's disease N=20	Culture of PBMCs with ovalbumin, IL-2 and IL-4 followed by cloning of ovalbumin-specific T cells	X-Vivo 15 IL-2 (200IU/ml) Anti-CD3/anti-CD28 beads (1:1) Ova-Tregs selected based on ovalbumin-specific IL-10 production 12 to 15 weeks	1x10 <sup>6</sup> -1x10 <sup>9</sup>	Well-tolerated; dose-related efficacy
Bluestone <i>et al.</i> (2015)	Safety in adults with type 1 diabetes (N=14)	FACS-based sorting of CD4 <sup>+</sup> CD25 <sup>hi</sup> CD127 <sup>lo</sup> cells	X-Vivo 15 with 10% human AB serum and deuterated glucose IL-2 (300IU/ml) Anti-CD3/anti-CD28 beads (1:1) 14 days	0.05x10 <sup>8</sup> -26x10 <sup>8</sup>	Well-tolerated, no significant adverse events. Stable C-peptide levels and insulin use in recipients for up to two years post infusion
Mathew <i>et al.</i> (2018)	Safety in living donor kidney transplant N=9	CliniMACS plus GMP enrichment system (Miltenyi)	IL-2 (1000IU/ml) MACS © GMP expansion beads 1:1-4:1 3 weeks	0.5-5x10 <sup>9</sup>	Well-tolerated, no infections or rejection up to two years post transplant

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

**Table 1: Summary of clinicaltrials.gov listings for reported trials using *in vitro* expanded regulatory T cell (Treg) therapy**

**Graft vs. host disease (GvHD):**

The risk of developing GvHD following haematopoietic stem cell transplantation (HSCT) is associated with low numbers of Tregs in the periphery,<sup>106</sup> and *in vivo* expansion of Tregs post-HSCT using low dose IL-2 has demonstrated efficacy against GvHD.<sup>107,108</sup> Studies in mice involving infusion of cultured CD4<sup>+</sup>CD25<sup>+</sup> T cells resulted in a significantly reduced GvHD phenotype,<sup>109</sup> and in humans it was found that infusion of freshly isolated donor Tregs given at the same time as haplotype mismatched HSCT prevented the development of GvHD.<sup>110</sup> Five trials of *ex vivo* expanded Tregs have to date involved small numbers of patients only, but suggest therapy can prevent or delay the onset of chronic GvHD<sup>111,112</sup>. Treg therapy seems to be effective only in the chronic form of GvHD, but this may be because of the time requirements to expand the cellular product which makes it difficult to administer in a timely manner in acute GvHD.<sup>113</sup>

**Solid organ transplant:**

Adoptive Treg therapy has been trialled following renal and liver transplantation, with the aim of inducing tolerance to the allograft and reducing the burden of long-term immunosuppression.<sup>114</sup> Tregs have been shown to control immune responsiveness to alloantigens and contribute to ‘operational tolerance’ in preclinical transplantation models.<sup>115,116</sup> Recipient-derived Tregs expanded for direct and indirect pathway allospecificity *in vitro* were able to mediate effective protection against acute and chronic rejection in skin and heart allografts in mice,<sup>117</sup> and could be used to induce tolerance of a murine skin transplant following thymectomy and T cell depletion.<sup>118</sup> In these models, alloantigen reactive Tregs were more effective at preventing graft rejection than polyclonally expanded Tregs<sup>104</sup>.

A phase I study in renal transplantation recruited nine living donor transplant recipients, and used the product of leukapheresis as the basis for *ex vivo* expansion of polyclonal

autologous Tregs.<sup>114</sup> Alemtuzumab was given at induction to achieve lymphodepletion, on the basis of previous experiments suggesting a reduction in circulating Tregs worked synergistically with Treg infusion to prolong allograft survival.<sup>116</sup> Recipients were switched from traditional immunosuppression with tacrolimus, which blocks IL-2 production, to sirolimus (rapamycin), which has Treg promoting activity.<sup>119</sup>

An enhanced suppressive ability of the expanded Tregs was demonstrated when compared to Tregs taken directly *ex vivo*.<sup>114</sup> There were no adverse infusion-related side effects, infections or rejection up to two years post-transplant, and there was a 5-20 fold increase in the number of circulating Tregs seen up to one year post-transplant. Transplant biopsies taken at three months did not show rejection and recipients had not developed peripheral donor-specific antibodies. An additional important outcome from trials in transplantation is that they have demonstrated that it is possible to expand Tregs from immunocompromised patients.<sup>120</sup>

A trial of Treg immunotherapy in liver transplantation is currently underway.<sup>121</sup> This is predicated on the observation that when liver allografts in mice were infiltrated with Tregs, loss of Treg numbers was associated with a loss of tolerance.<sup>122</sup> Increased frequencies of Tregs are also seen in human subjects who acquire 'operational tolerance' to their liver transplant.<sup>123</sup>

### **Type 1 diabetes mellitus:**

The development of T1DM is associated with deficits in the number and suppressive activity of Tregs.<sup>124</sup> Accelerated diabetes onset is seen in both scurfy mice<sup>29</sup> and children with IPEX,<sup>125</sup> highlighting the role of Tregs in protecting pancreatic islet cells from destruction. Tregs have been implicated in the pathogenesis of diabetes in the non-obese diabetic (NOD) mouse model,<sup>126,127</sup> and anti-CD3 antibodies have been efficacious in the treatment of diabetes in both mouse<sup>128,129</sup> and human trials.<sup>130,131</sup> Subjects exhibited lower insulin requirements and higher C-peptide levels at least 18 months after a short course of intravenous treatment, with evidence of anti-CD3 treatment inducing expansion of a CD4<sup>+</sup>CD25<sup>+</sup> T cell population.<sup>129</sup> A trial of ten children treated with expanded polyclonal Tregs



within two months of their diagnosis demonstrated statistically lower insulin requirements and C peptide levels compared with matched controls up to six months post infusion, with two patients remaining insulin-independent.<sup>124</sup> There were no serious adverse events up to one year following infusion.

In a phase I open-label trial of 14 adult patients infused with *ex vivo* expanded Tregs in escalating doses, 7 of 14 patients had stable C peptide levels and insulin use for up to two years following infusion.<sup>101</sup> However, the study was not powered to detect significant clinical improvement. There were no infusion reactions or therapy-related serious adverse events. Phenotypic analysis of the cell product after expansion and after infusion identified stable surface marker expression, demonstrating that the infused Tregs did not acquire a pathological phenotype. High throughput TCR- $\beta$  sequencing analysis indicated that expanded Tregs retained a high degree of diversity.

Adoptively transferred Tregs were tagged by labelling the deoxyribose moiety of replicating DNA during expansion *ex vivo*, through addition of deuterated [6,6-<sup>2</sup>H<sub>2</sub>] glucose to Treg culture throughout the 14 day expansion period.<sup>101</sup> Patient samples were analysed by gas chromatography mass spectrometry (GC-MS) for deuterium enrichment to create pharmacokinetic curves. Adoptively transferred T cell numbers peaked at two weeks following infusion, but were still detectable at up to 25% of the peak level at one year in peripheral blood. Significantly, deuterium labelling was never found in non-Tregs, indicating the stability of infused Tregs. However, due to the nature of this study, the stability of these cells was not assessed within the target tissue.

**TREG THERAPY IN INFLAMMATORY BOWEL DISEASE:**

A local imbalance between Treg and Teff responses plays a key role in the development of gut inflammation in IBD.<sup>8</sup> T cell gut homing is mediated by specific interaction between integrin  $\alpha 4\beta 7$  and its ligand MAdCAM-1.<sup>132,133</sup> Several groups have shown that transfer of Tregs into mice leads to clinical and histological improvement in colitis,<sup>14,134,135</sup> and rapamycin-expanded Tregs ameliorated established colitis in a SCID mouse model.<sup>136</sup> Polyclonality of the TCR is likely to be an important requirement for Tregs to maintain

intestinal homeostasis *in vivo*. Mice which express a restricted TCR repertoire develop spontaneous colitis due to a loss of tolerance to intestinal microbiota.<sup>137</sup>

Several groups have demonstrated that it is feasible to extract Tregs from patients, and expand them *in vitro* under GMP conditions, including from subjects receiving thiopurines and anti-TNF $\alpha$  medications.<sup>98,100,103,138</sup> Even after prolonged culture, these Tregs maintained Foxp3 expression and demonstrated enhanced suppression of autologous T cells. Uncertainty regarding the potential for adoptively transferred Tregs to express IL-17 and exacerbate CD lesions is a concern. However, the administration of pro-inflammatory cytokines (IL-1, IL-2, IL-6, IL-21, IL-21 and TGF- $\beta$ ) failed to induce IL-17 production by CD45RA<sup>+</sup> expanded Tregs *in vitro*.<sup>100</sup>

#### **Antigen specific vs. polyclonal Treg cell products for Crohn's disease:**

No antigens have yet been verified as causal in CD. Attempts have been made to identify shared TCRs between CD sufferers with the aim of discovering target antigens.<sup>139–141</sup> This work has observed that the CD4 TCR repertoires are significantly more diverse in patients with CD and UC than healthy controls.<sup>142</sup> This may be explained by GI barrier disruption increasing the number of antigen presentation events in comparison to a healthy gut. Resolving a target from the GI peptidome is challenging due to the heterogenous nature of the environment. Developments in the understanding of non-conventional epitopes are also increasing the magnitude and complexity of the peptidome itself.<sup>143</sup> In the absence of a known target, the broad reactivity of a polyclonal Treg product may be advantageous, as the cell product will recognise millions of putative epitopes, increasing the likelihood of TCR engagement and subsequent Treg activation. Sequencing of isolated Tregs from GI biopsies post transfer may yield novel targets, upon which chimeric antigen receptor technology could be readily implemented.<sup>144</sup>

**For Treg therapy to be effective in IBD, expanded Tregs must have the ability to home to the gut.**<sup>145</sup> A French group reported the results of an open label multicentre phase I/IIa trial of ovalbumin-specific Tregs in 20 patients with refractory CD.<sup>146</sup> Ovalbumin is a common food antigen, and is not implicated in intestinal inflammation in animal models or in patients with

1  
2  
3 585 CD. Its distribution along the digestive tract can be used to activate Tregs locally. In the  
4  
5 586 study, this was facilitated through ingestion of meringue cakes by subjects.<sup>146</sup>  
6  
7 587  
8  
9 588 The cell product was cultured in the presence of ovalbumin, and trial subjects received a  
10  
11 589 dose of 10<sup>6</sup>-10<sup>9</sup> Tregs.<sup>146</sup> Patients enrolled in the study had at least moderately active CD,  
12  
13 590 with a Crohn's Disease Activity Index (CDAI) greater than or equal to 220 within six months  
14  
15 591 of screening, and a washout period was required for immunosuppression and anti-TNFα  
16  
17 592 therapy. The infusion was well tolerated, with mild GI symptoms and CD flares being the  
18  
19 593 most commonly reported adverse effects. Two patients experienced thrombotic events, but  
20  
21 594 these are known to occur more frequently in inflammatory conditions including active CD.<sup>147</sup>  
22  
23 595 Eight (40%) patients had a significant CDAI response at weeks five and eight after treatment,  
24  
25 596 with two patients experiencing sustained remission. Overall, the results suggested good  
26  
27 597 tolerability in this disease group with possible signals of efficacy.  
28  
29 598  
29 599 In the absence of a known antigen, other methods must be used to direct the Tregs to the  
30  
31 600 areas of inflammation. A recent study has shown that a highly specific retinoic acid receptor  
32  
33 601 α (RARα) agonist induces expression of Integrin α4β7 (the ligand of MAdCAM-1) on the Treg  
34  
35 602 surface. Adoptive transfer of RARα agonist-treated Tregs leads to improved Treg trafficking  
36  
37 603 to gut tissue in a humanised mouse model of colitis.<sup>100</sup> Supporting this mechanism for  
38  
39 604 resolving inflammation, another group have demonstrated that DCs can be engineered *de*  
40  
41 605 *novo* to produce high concentrations of RA.<sup>148</sup> When transferred to mice, the RA-secreting  
42  
43 606 DCs were able to augment the expression of Foxp3 and the gut-homing receptor CCR9 in  
44  
45 607 native Tregs with the subsequent suppression of colitis.  
46  
47 608  
47 609 The RARα agonist treated cell product forms the basis of the TRIBUTE trial (ClinicalTrials.gov  
48  
49 610 Identifier: NCT03185000), a double-blinded placebo-controlled phase I/IIa trial of adoptive  
50  
51 611 Treg therapy in CD.  
52  
53 612  
54 613 **FUTURE DEVELOPMENTS IN TREG THERAPY:**  
55  
56 614  
57  
58 615 The potential therapeutic benefits of adoptive cell therapy are being explored in numerous  
59  
60 616 autoimmune conditions. In SLE, adoptive transfer of *ex vivo* expanded Tregs in mice delayed

the onset of renal complications and prolonged survival,<sup>149,150</sup> and a pilot study of low dose IL-2 in 37 patients led to increased circulating peripheral Treg numbers and decreased SLE disease activity scores.<sup>151</sup> Adoptive Treg transfer in a single patient with cutaneous lupus did not lead to clinical benefit, but increased percentages of highly activated Tregs were identified in biopsies taken from diseased skin.<sup>152</sup> Treg accumulation in skin was associated with a marked attenuation of IFN- $\gamma$ , which was more pronounced relative to peripheral blood.

Preliminary results from mouse models suggest a role for Treg therapy in conditions as diverse as pemphigus vulgaris,<sup>153</sup> autoimmune hepatitis,<sup>154</sup> multiple sclerosis,<sup>113</sup> asthma, and allergy, in which antigen-specific Tregs may represent a viable therapeutic option.<sup>155,156</sup>

Many ongoing challenges exist for the advancement of Treg therapy. Uncertainties remain about the optimal timing of *ex vivo* Treg expansion, and whether IL-2 administration would be a useful adjunct to support a Treg population *in vivo*.<sup>101,107</sup> In addition, concomitant treatment of autoimmune disease with immunosuppressive drugs may affect the function of adoptively transferred cells.<sup>95</sup>

The optimal dosing strategy for Treg therapy also remains unclear, although data tracking the survival of deuterium-labelled Tregs *in vivo* could be invaluable in informing a suitable dosing regimen.<sup>101</sup> A two-phase decay in numbers of deuterium-labelled Tregs has been seen, with 75% of the peak level lost at three months. However, levels stabilised at one year, with up to 25% of peak Treg numbers remaining in the peripheral circulation. The decrease in labelled Tregs may represent cell death, trafficking to lymphoid tissue and sites of inflammation, or proliferation of the Treg compartment leading to dilution of deuterium enrichment. Reassuringly, at no point during the trial was deuterium detected in cell populations other than Tregs, suggesting a stable phenotype *in vivo*.<sup>101</sup>

Tracking of TCR clonotypes may also provide useful data on Treg kinetics and dispersal. Analysis of the TCR repertoire has suggested that the kinetics of transferred Tregs in peripheral blood varies significantly between individuals<sup>157</sup>. In a descriptive study, the TCR V $\alpha$  chain was sequenced in two patients receiving donor Treg infusion<sup>157</sup>. Treg therapy

1  
2  
3 649 altered the patients' peripheral TCR repertoire considerably towards that of the infused cell  
4  
5 650 product, but to different degrees in each patient. Importantly, the degree of alteration of  
6  
7 651 the TCR repertoire appeared to correlate with clinical response. This suggests that  
8  
9 652 monitoring TCR repertoires following adoptive cell transfer may provide clinically  
10  
11 653 meaningful information.

12 654  
13  
14 655

15  
16 656 **CONCLUSION:**

17  
18 657

19  
20 658 There is now robust evidence of the therapeutic potential of Treg therapy in Crohn's  
21  
22 659 disease. Trials in multiple autoimmune diseases and results from use of ovalbumin-specific  
23  
24 660 Tregs in IBD show promising early signs of efficacy. The safety signal is reassuring, with  
25  
26 661 evidence that the adoptively transferred Treg phenotype is stable *in vivo*. Results from  
27  
28 662 deuterium labelling suggest that infused Tregs may be able to exert a long-lasting systemic  
29  
30 663 effect with labelled cells detectable up to a year after infusion. It is hoped that upcoming  
31  
32 664 early phase clinical trials in patients with Crohn's disease will inform safety, dosing, and Treg  
33  
34 665 kinetics and dispersal allowing further development of a novel therapeutic option in this  
35  
36 666 hard-to-treat population.

36 667

37  
38 668 **FIGURE LEGEND:**

39  
40 669

41  
42 670 **Figure 1. Mechanisms of Treg mediated suppression**

43  
44 671 Tregs utilize a multitude of mechanisms to promote a tolerogenic microenvironment and  
45  
46 672 tissue repair. (A) Secretion of the anti-inflammatory cytokines, IL-10, TGF- $\beta$  and IL-35, not  
47  
48 673 only inhibit Teff proliferation, but also suppress Th1 and Th17 effector function, both of  
49  
50 674 which are key mediators of IBD. (B) Tregs express the high affinity IL-2 receptor  $\alpha$ -chain  
51  
52 675 (CD25) consuming local IL-2 with greater affinity than effector cells. Teffs which are 'starved'  
53  
54 676 of IL-2 exhibit restricted proliferation and undergo apoptosis. (C) Tregs co-expressing CD39  
55  
56 677 and CD73 disrupt metabolic processes in effector cells by converting ATP into peri-cellular  
57  
58 678 adenosine, a potent inhibitor of Teff function. Additionally, adenosine stimulates TGF- $\beta$   
59  
60 679 production, promoting development of pTregs. (D) Tregs are capable of secreting perforin,

granzyme B and galectin-1 which are directly cytotoxic against Teffs. Activated Tregs also express TRAIL, inducing apoptosis of Teffs through the TRAIL/DR5 pathway. (E) Expression of CTLA-4 degrades DC-derived CD80 and CD86 leading to impaired CD28-mediated co-stimulation of T cells. DC function is further inhibited through the interaction of Treg-derived TIGIT and CD155 on DCs. This induces IL-10 production and suppresses IL-12. (F) In response to alarmins, Tregs produce AREG, an important regulator of tissue repair and regeneration. AREG, amphiregulin; ATP, adenosine tri-phosphate; CTLA-4, cytotoxic T lymphocyte associated protein 4; DC, dendritic cell; DR5, death receptor 5; IBD, inflammatory bowel disease; pTregs, peripheral regulatory T cells; Teff, T effector lymphocyte; TGF- $\beta$ , transforming growth factor beta; Th1, T helper 1 cell; Th17, T helper 17 cell; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; TRAIL, TNF-related apoptosis-inducing ligand; Treg, regulatory T cell. Figure generated using BioRender© illustration software.

#### ACKNOWLEDGEMENTS:

Support was provided by Litwin IBD Pioneers Funding Program at the Crohn's and Colitis Foundation (grant number 504039), the Freemason's Grand Charity (grant number SPG00268) and the Rosetree's Trust (A1219). OO is funded by an MRC Trust Clinical Research Training Fellowship (MRS00033X/1). This work was also supported by MRC Grants to GL (MR/R001413/1) and to PI (MR/T005564/1). The authors also wish to acknowledge financial support from the Department of Health via the National Institute for Health (NIHR) comprehensive Biomedical Research Centre award to Guy's & St Thomas' NHS Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health.

#### COMPETING INTERESTS:

PI is the Chief Investigator and GL is the Chief Scientific Investigator on the MRC-funded TRIBUTE trial of regulatory T cell immunotherapy in Crohn's disease (ClinicalTrials.gov NCT03185000).

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

712  
713

Confidential: For Review Only



## References:

1. Kaplan, G. G. The global burden of IBD: from 2015 to 2025. *Nat. Rev. Gastroenterol. Hepatol.* **12**, 720–727 (2015).
2. Burisch, J., Jess, T., Martinato, M., Lakatos, P. L. & ECCO -EpiCom. The burden of inflammatory bowel disease in Europe. *J. Crohn's Colitis* **7**, 322–337 (2013).
3. Neurath, M. F. Current and emerging therapeutic targets for IBD. *Nat. Rev. Gastroenterol. Hepatol.* **14**, 269–278 (2017).
4. Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat. Immunol.* **4**, 330–336 (2003).
5. Hori, S., Nomura, T. & Sakaguchi, S. Control of Regulatory T Cell Development by the Transcription Factor Foxp3. *Science* (80-. ). **299**, 1057–1061 (2003).
6. Khattari, R., Cox, T., Yasayko, S.-A. & Ramsdell, F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat. Immunol.* **4**, 337–342 (2003).
7. Liu, W. *et al.* CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J. Exp. Med.* **203**, 1701–11 (2006).
8. Maul, J. *et al.* Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease. *Gastroenterology* **128**, 1868–78 (2005).
9. Fantini, M. C. *et al.* Smad7 Controls Resistance of Colitogenic T Cells to Regulatory T Cell-Mediated Suppression. *Gastroenterology* **136**, 1308-1316.e3 (2009).
10. Nishizuka, Y. & Sakakura, T. Thymus and reproduction: sex-linked dysgenesis of the gonad after neonatal thymectomy in mice. *Science* **166**, 753–5 (1969).
11. Sakaguchi, S., Takahashi, T. & Nishizuka, Y. Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis. *J. Exp. Med.* **156**, 1577–86 (1982).
12. Sakaguchi, S., Takahashi, T. & Nishizuka, Y. Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis. *J. Exp. Med.* **156**, 1577–86 (1982).
13. Fowell, D. & Mason, D. Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. Characterization of the CD4+ T cell subset that inhibits this autoimmune potential. *J. Exp. Med.* **177**, 627–36 (1993).
14. Mottet, C., Uhlig, H. H. & Powrie, F. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. *J. Immunol.* **170**, 3939–43 (2003).
15. Chen, W. *et al.* Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J. Exp. Med.* **198**, 1875–86 (2003).
16. Sun, C.-M. *et al.* Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J. Exp. Med.* **204**, 1775–85 (2007).
17. Kanamori, M., Nakatsukasa, H., Okada, M., Lu, Q. & Yoshimura, A. Induced Regulatory T Cells: Their Development, Stability, and Applications. *Trends Immunol.* **37**, 803–811 (2016).
18. Coombes, J. L. *et al.* A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J. Exp. Med.* **204**, 1757–64 (2007).
19. Ruane, D. T. & Lavelle, E. C. The Role of CD103+ Dendritic Cells in the Intestinal Mucosal Immune System. *Front. Immunol.* **2**, 25 (2011).
20. del Rio, M.-L., Bernhardt, G., Rodriguez-Barbosa, J.-I. & Förster, R. Development and

- functional specialization of CD103+ dendritic cells. *Immunol. Rev.* **234**, 268–281 (2010).
21. Matsuno, H. *et al.* CD103+ Dendritic Cell Function Is Altered in the Colons of Patients with Ulcerative Colitis. *Inflamm. Bowel Dis.* **23**, 1524–1534 (2017).
  22. Nutsch, K. *et al.* Rapid and Efficient Generation of Regulatory T Cells to Commensal Antigens in the Periphery. *Cell Rep.* **17**, 206–220 (2016).
  23. Kanamori, M., Nakatsukasa, H., Okada, M., Lu, Q. & Yoshimura, A. Induced Regulatory T Cells: Their Development, Stability, and Applications. *Trends Immunol.* **37**, 803–811 (2016).
  24. Baecher-Allan, C., Brown, J. A., Freeman, G. J. & Hafler, D. A. CD4+CD25<sup>high</sup> regulatory cells in human peripheral blood. *J. Immunol.* **167**, 1245–53 (2001).
  25. Gavin, M. A. *et al.* Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* **445**, 771–775 (2007).
  26. Martin, F., Ladoire, S., Mignot, G., Apetoh, L. & Ghiringhelli, F. Human FOXP3 and cancer. *Oncogene* **29**, 4121–4129 (2010).
  27. Brunkow, M. E. *et al.* Disruption of a new forkhead/winged-helix protein, scurfy, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* **27**, 68–73 (2001).
  28. Bennett, C. L. *et al.* The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* **27**, 20–21 (2001).
  29. Wildin, R. S. *et al.* X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat. Genet.* **27**, 18–20 (2001).
  30. Husebye, E. S., Anderson, M. S. & Kämpe, O. Autoimmune Polyendocrine Syndromes. *N. Engl. J. Med.* **378**, 1132–1141 (2018).
  31. Weiss, J. M. *et al.* Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3<sup>+</sup> T reg cells. *J. Exp. Med.* **209**, 1723 (2012).
  32. Sefik, E. *et al.* Individual intestinal symbionts induce a distinct population of RORγ<sup>+</sup> regulatory T cells. *Science (80-. )*. **349**, 993–7 (2015).
  33. Yang, B.-H. *et al.* Foxp3<sup>+</sup> T cells expressing RORγ<sup>t</sup> represent a stable regulatory T-cell effector lineage with enhanced suppressive capacity during intestinal inflammation. *Mucosal Immunol.* **9**, 444–457 (2016).
  34. Whibley, N., Tucci, A. & Powrie, F. Regulatory T cell adaptation in the intestine and skin. *Nature Immunology* **20**, 386–396 (2019).
  35. Kim, K. S. *et al.* Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine. *Science (80-. )*. **351**, 858–863 (2016).
  36. Ohnmacht, C. *et al.* MUCOSAL IMMUNOLOGY. The microbiota regulates type 2 immunity through RORγ<sup>t</sup> T cells. *Science* **349**, 989–93 (2015).
  37. Schiering, C. *et al.* The alarmin IL-33 promotes regulatory T-cell function in the intestine. *Nature* **513**, 564–568 (2014).
  38. Lindley, S. *et al.* Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. *Diabetes* **54**, 92–9 (2005).
  39. Viglietta, V., Baecher-Allan, C., Weiner, H. L. & Hafler, D. A. Loss of functional suppression by CD4+CD25<sup>+</sup> regulatory T cells in patients with multiple sclerosis. *J. Exp. Med.* **199**, 971–9 (2004).

40. Bonelli, M. *et al.* Quantitative and qualitative deficiencies of regulatory T cells in patients with systemic lupus erythematosus (SLE). *Int. Immunol.* **20**, 861–868 (2008).
41. Thiruppathi, M. *et al.* Functional defect in regulatory T cells in myasthenia gravis. *Ann. N. Y. Acad. Sci.* **1274**, 68–76 (2012).
42. van Roon, J. A. G., Hartgring, S. A. Y., van der Wurff-Jacobs, K. M. G., Bijlsma, J. W. J. & Lafeber, F. P. J. G. Numbers of CD25+Foxp3+ T cells that lack the IL-7 receptor are increased intra-articularly and have impaired suppressive function in RA patients. *Rheumatology* **49**, 2084–2089 (2010).
43. Fantini, M. C. *et al.* Smad7 Controls Resistance of Colitogenic T Cells to Regulatory T Cell-Mediated Suppression. *Gastroenterology* **136**, 1308–1316.e3 (2009).
44. Takahashi, T. *et al.* Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int. Immunol.* **10**, 1969–80 (1998).
45. Thornton, A. M. & Shevach, E. M. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* **188**, 287–96 (1998).
46. Rubtsov, Y. P. *et al.* Regulatory T Cell-Derived Interleukin-10 Limits Inflammation at Environmental Interfaces. *Immunity* **28**, 546–558 (2008).
47. Vignali, D. A. A., Collison, L. W. & Workman, C. J. How regulatory T cells work. *Nat. Rev. Immunol.* **8**, 523–532 (2008).
48. Levine, A. G., Arvey, A., Jin, W. & Rudensky, A. Y. Continuous requirement for the TCR in regulatory T cell function. *Nat. Immunol.* **15**, 1070–1078 (2014).
49. Powrie, F. *et al.* Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity* **1**, 553–62 (1994).
50. Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L. & Powrie, F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* **190**, 995–1004 (1999).
51. Chaudhry, A. *et al.* Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity* **34**, 566–78 (2011).
52. Ahern, P. P., Izcue, A., Maloy, K. J. & Powrie, F. The interleukin-23 axis in intestinal inflammation. *Immunol. Rev.* **226**, 147–159 (2008).
53. Engelhardt, K. R. & Grimbacher, B. IL-10 in humans: Lessons from the Gut, IL-10/IL-10 receptor deficiencies, and IL-10 polymorphisms. *Curr. Top. Microbiol. Immunol.* **380**, 1–18 (2014).
54. Sun, C.-M. *et al.* Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J. Exp. Med.* **204**, 1775–85 (2007).
55. Nakamura, K. *et al.* TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice. *J. Immunol.* **172**, 834–42 (2004).
56. Li, M. O., Wan, Y. Y. & Flavell, R. A. T Cell-Produced Transforming Growth Factor-β1 Controls T Cell Tolerance and Regulates Th1- and Th17-Cell Differentiation. *Immunity* **26**, 579–591 (2007).
57. Di Sabatino, A. *et al.* Blockade of transforming growth factor beta upregulates T-box transcription factor T-bet, and increases T helper cell type 1 cytokine and matrix metalloproteinase-3 production in the human gut mucosa. *Gut* **57**, 605–12 (2008).
58. Monteleone, G. *et al.* Blocking Smad7 restores TGF-beta1 signaling in chronic inflammatory bowel disease. *J. Clin. Invest.* **108**, 601–9 (2001).

- 855 59. Monteleone, G. *et al.* Mongersen, an Oral SMAD7 Antisense Oligonucleotide, and  
856 Crohn's Disease. *N. Engl. J. Med.* **372**, 1104–1113 (2015).
- 857 60. ClinicalTrials.gov. Efficacy and Safety Study of Mongersen (GED-0301) for the  
858 Treatment of Subjects With Active Crohn's Disease.
- 859 61. Collison, L. W. *et al.* The inhibitory cytokine IL-35 contributes to regulatory T-cell  
860 function. *Nature* **450**, 566–569 (2007).
- 861 62. Collison, L. W. *et al.* IL-35-mediated induction of a potent regulatory T cell population.  
862 *Nat. Immunol.* **11**, 1093–1101 (2010).
- 863 63. Wirtz, S., Billmeier, U., Mchedlidze, T., Blumberg, R. S. & Neurath, M. F. Interleukin-35  
864 mediates mucosal immune responses that protect against T-cell-dependent colitis.  
865 *Gastroenterology* **141**, 1875–86 (2011).
- 866 64. Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. Regulatory T Cells and Immune  
867 Tolerance. *Cell* **133**, 775–787 (2008).
- 868 65. Chinen, T. *et al.* An essential role for the IL-2 receptor in Treg cell function. *Nat.*  
869 *Immunol.* **17**, 1322–1333 (2016).
- 870 66. Abbas, A. K., Trotta, E., R Simeonov, D., Marson, A. & Bluestone, J. A. Revisiting IL-2:  
871 Biology and therapeutic prospects. *Sci. Immunol.* **3**, (2018).
- 872 67. Fan, M. Y. *et al.* Differential Roles of IL-2 Signaling in Developing versus Mature Tregs.  
873 *Cell Rep.* **25**, 1204-1213.e4 (2018).
- 874 68. Waldmann, T. A. The Multi-Subunit Interleukin-2 Receptor. *Annu. Rev. Biochem.* **58**,  
875 875–905 (1989).
- 876 69. de la Rosa, M., Rutz, S., Dorninger, H. & Scheffold, A. Interleukin-2 is essential for  
877 CD4+CD25+ regulatory T cell function. *Eur. J. Immunol.* **34**, 2480–2488 (2004).
- 878 70. Pandiyan, P., Zheng, L., Ishihara, S., Reed, J. & Lenardo, M. J. CD4+CD25+Foxp3+  
879 regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T  
880 cells. *Nat. Immunol.* **8**, 1353–1362 (2007).
- 881 71. Sharfe, N., Dadi, H. K., Shahar, M. & Roifman, C. M. Human immune disorder arising  
882 from mutation of the  $\alpha$  chain of the interleukin-2 receptor. *Proc. Natl. Acad. Sci.* **94**,  
883 3168–3171 (1997).
- 884 72. Caudy, A. A., Reddy, S. T., Chatila, T., Atkinson, J. P. & Verbsky, J. W. CD25 deficiency  
885 causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like  
886 syndrome, and defective IL-10 expression from CD4 lymphocytes. *J. Allergy Clin.*  
887 *Immunol.* **119**, 482–7 (2007).
- 888 73. Goudy, K. *et al.* Human IL2RA null mutation mediates immunodeficiency with  
889 lymphoproliferation and autoimmunity. *Clin. Immunol.* **146**, 248–261 (2013).
- 890 74. Caudy, A. A., Reddy, S. T., Chatila, T., Atkinson, J. P. & Verbsky, J. W. CD25 deficiency  
891 causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like  
892 syndrome, and defective IL-10 expression from CD4 lymphocytes. *J. Allergy Clin.*  
893 *Immunol.* **119**, 482–7 (2007).
- 894 75. Deaglio, S. *et al.* Adenosine generation catalyzed by CD39 and CD73 expressed on  
895 regulatory T cells mediates immune suppression. *J. Exp. Med.* **204**, 1257–65 (2007).
- 896 76. Zarek, P. E. *et al.* A2A receptor signaling promotes peripheral tolerance by inducing T-  
897 cell anergy and the generation of adaptive regulatory T cells. *Blood* **111**, 251–259  
898 (2008).
- 899 77. Kurtz, C. C. *et al.* Extracellular adenosine regulates colitis through effects on lymphoid  
900 and nonlymphoid cells. *Am. J. Physiol. Liver Physiol.* **307**, G338–G346 (2014).
- 901 78. Takahashi, T. *et al.* Immunologic self-tolerance maintained by CD25(+)CD4(+)



- regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med.* **192**, 303–10 (2000).
79. Qureshi, O. S. *et al.* Trans-endocytosis of CD80 and CD86: a molecular basis for the cell extrinsic function of CTLA-4. *Science* (80-. ). **332**, 600 (2011).
  80. Oderup, C., Cederbom, L., Makowska, A., Cilio, C. M. & Ivars, F. Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. *Immunology* **118**, 240–249 (2006).
  81. Wing, K. *et al.* CTLA-4 control over Foxp3+ regulatory T cell function. *Science* **322**, 271–5 (2008).
  82. Kuehn, H. S. *et al.* Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. *Science* (80-. ). **345**, 1623–1627 (2014).
  83. Lo, B. *et al.* Patients with LRBA deficiency show CTLA4 loss and immune dysregulation responsive to abatacept therapy. *Science* (80-. ). **349**, 436–40 (2015).
  84. Lopez-Herrera, G. *et al.* Deleterious mutations in LRBA are associated with a syndrome of immune deficiency and autoimmunity. *Am. J. Hum. Genet.* **90**, 986–1001 (2012).
  85. Gamez-Diaz, L. G. *et al.* The extended phenotype of LPS-responsive beige-like anchor protein (LRBA) deficiency. *J. Allergy Clin. Immunol.* **137**, 223–230 (2016).
  86. Yu, X. *et al.* The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat. Immunol.* **10**, 48–57 (2009).
  87. Joller, N. *et al.* Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. *Immunity* **40**, 569–81 (2014).
  88. Grossman, W. J. *et al.* Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood* **104**, 2840–2848 (2004).
  89. Ren, X. *et al.* Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4+CD25+ regulatory T cells. *Cell Death Differ.* **14**, 2076–2084 (2007).
  90. Garin, M. I. *et al.* Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. *Blood* **109**, 2058–2065 (2007).
  91. Arpaia, N. *et al.* A Distinct Function of Regulatory T Cells in Tissue Protection. *Cell* **162**, 1078–89 (2015).
  92. Beltrán, C. J. *et al.* Characterization of the novel ST2/IL-33 system in patients with inflammatory bowel disease. *Inflamm. Bowel Dis.* **16**, 1097–1107 (2010).
  93. Kobori, A. *et al.* Interleukin-33 expression is specifically enhanced in inflamed mucosa of ulcerative colitis. *J. Gastroenterol.* **45**, 999–1007 (2010).
  94. Schiering, C. *et al.* The alarmin IL-33 promotes regulatory T-cell function in the intestine. *Nature* **513**, 564–568 (2014).
  95. Esensten, J. H., Muller, Y. D., Bluestone, J. A. & Tang, Q. Regulatory T-cell therapy for autoimmune and autoinflammatory diseases: The next frontier. *J. Allergy Clin. Immunol.* **142**, 1710–1718 (2018).
  96. Sagoo, P., Lombardi, G. & Lechler, R. I. Regulatory T cells as therapeutic cells. doi:10.1097/MOT.0b013e328317a476
  97. Apostolou, I. & Von Boehmer, H. In vivo instruction of suppressor commitment in naive T cells. *J. Exp. Med.* **199**, 1401–1408 (2004).
  98. Hoffmann, P., Eder, R., Kunz-Schughart, L. A., Andreesen, R. & Edinger, M. Large-scale in vitro expansion of polyclonal human CD4 CD25 high regulatory T cells. (2004).

- doi:10.1182/blood-2004-01-0086
99. Gravano, D. M. & Vignali, D. A. A. The battle against immunopathology: infectious tolerance mediated by regulatory T cells. *Cell. Mol. Life Sci.* **69**, 1997–2008 (2012).
  100. Canavan, J. B. *et al.* Developing in vitro expanded CD45RA+regulatory T cells as an adoptive cell therapy for Crohn's disease. *Gut* **65**, 584–594 (2016).
  101. Bluestone, J. A. *et al.* Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Sci. Transl. Med.* **7**, 315ra189 (2015).
  102. Trzonkowski, P., Szaryńska, M., Myśliwska, J. & Myśliwski, A. Ex vivo expansion of CD4<sup>+</sup> CD25<sup>+</sup> T regulatory cells for immunosuppressive therapy. *Cytom. Part A* **75A**, 175–188 (2009).
  103. Golovina, T. N. *et al.* Retinoic Acid and Rapamycin Differentially Affect and Synergistically Promote the Ex Vivo Expansion of Natural Human T Regulatory Cells. *PLoS One* **6**, e15868 (2011).
  104. Putnam, A. L. *et al.* Clinical Grade Manufacturing of Human Alloantigen-Reactive Regulatory T Cells for Use in Transplantation. *Am. J. Transplant.* **13**, 3010–3020 (2013).
  105. Tresoldi, E. *et al.* Stability of human rapamycin-expanded CD4+CD25+ T regulatory cells. *Haematologica* **96**, 1357–1365 (2011).
  106. Trzonkowski, P. *et al.* Differences in Kinetics of Donor Lymphoid Cells in Response to G-CSF Administration May Affect the Incidence and Severity of Acute GvHD in Respective HLA-Identical Sibling Recipients. *Med. Oncol.* **21**, 81–94 (2004).
  107. Kennedy-Nasser, A. A. *et al.* Ultra low-dose IL-2 for GVHD prophylaxis after allogeneic hematopoietic stem cell transplantation mediates expansion of regulatory T cells without diminishing antiviral and antileukemic activity. *Clin. Cancer Res.* **20**, 2215–25 (2014).
  108. Koreth, J. *et al.* Interleukin-2 and Regulatory T Cells in Graft-versus-Host Disease. *N. Engl. J. Med.* **365**, 2055–2066 (2011).
  109. Taylor, P. A. *et al.* The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* **99**, 3493–9 (2002).
  110. Di Ianni, M. *et al.* Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* **117**, 3921–8 (2011).
  111. Trzonkowski, P. *et al.* First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127<sup>−</sup> T regulatory cells. *Clin. Immunol.* **133**, 22–26 (2009).
  112. Martelli, M. F. *et al.* HLA-haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents acute leukemia relapse. *Blood* **124**, 638–644 (2014).
  113. Gliwiński, M., Iwaszkiewicz-Grześ, D. & Trzonkowski, P. Cell-Based Therapies with T Regulatory Cells. *BioDrugs* **31**, 335–347 (2017).
  114. Mathew, J. M. *et al.* A Phase I Clinical Trial with Ex Vivo Expanded Recipient Regulatory T cells in Living Donor Kidney Transplants. *Sci. Rep.* **8**, 7428 (2018).
  115. Xia, G., He, J., Zhang, Z. & Leventhal, J. R. Targeting Acute Allograft Rejection by Immunotherapy With Ex Vivo-Expanded Natural CD4+CD25+ Regulatory T Cells. *Transplantation* **82**, 1749–1755 (2006).
  116. Xia, G., He, J. & Leventhal, J. R. Ex Vivo-Expanded Natural CD4+CD25+ Regulatory T Cells Synergize With Host T-Cell Depletion to Promote Long-Term Survival of

- Allografts. *Am. J. Transplant.* **8**, 298–306 (2008).
117. Joffre, O. *et al.* Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes. *Nat. Med.* **14**, 88–92 (2008).
  118. Tosello, V. *et al.* Differential expression of CCR7 defines two distinct subsets of human memory CD4+CD25+ Tregs. *Clin. Immunol.* **126**, 291–302 (2008).
  119. Gallon, L. *et al.* Differential Effects of Calcineurin and Mammalian Target of Rapamycin Inhibitors on Alloreactive Th1, Th17, and Regulatory T Cells. *Transplantation* **99**, 1774–1784 (2015).
  120. Chandran, S. *et al.* Polyclonal Regulatory T Cell Therapy for Control of Inflammation in Kidney Transplants. *Am. J. Transplant* **17**, 2945–2954 (2017).
  121. Safinia, N. *et al.* Successful expansion of functional and stable regulatory T cells for immunotherapy in liver transplantation. *Oncotarget* **7**, 7563–77 (2016).
  122. Li, W. *et al.* The Role of Foxp3+ Regulatory T Cells in Liver Transplant Tolerance. *Transplant. Proc.* **38**, 3205–3206 (2006).
  123. Li, Y. *et al.* Analyses of Peripheral Blood Mononuclear Cells in Operational Tolerance After Pediatric Living Donor Liver Transplantation. *Am. J. Transplant.* **4**, 2118–2125 (2004).
  124. Marek-Trzonkowska, N. *et al.* Administration of CD4+CD25highCD127- Regulatory T Cells Preserves  $\beta$ -Cell Function in Type 1 Diabetes in Children. *Diabetes Care* **35**, 1817–1820 (2012).
  125. Barzaghi, F. *et al.* Long-term follow-up of IPEX syndrome patients after different therapeutic strategies: An international multicenter retrospective study. *J. Allergy Clin. Immunol.* **141**, 1036–1049.e5 (2018).
  126. Holohan, D. R., Van Gool, F. & Bluestone, J. A. Thymically-derived Foxp3+ regulatory T cells are the primary regulators of type 1 diabetes in the non-obese diabetic mouse model. *PLoS One* **14**, e0217728 (2019).
  127. Fouser, G. *et al.* Following the fate of one insulin-reactive CD4 T cell: Conversion into Tregs and Tregs in the periphery controls diabetes in NOD mice. *Diabetes* **61**, 1169–1179 (2012).
  128. Chatenoud, L., Primo, J. & Bach, J. F. CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice. *J. Immunol.* **158**, 2947–54 (1997).
  129. Belghith, M. *et al.* TGF- $\beta$ -dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat. Med.* **9**, 1202–8 (2003).
  130. Herold, K. C. *et al.* Activation of human T cells by FcR nonbinding anti-CD3 mAb, hOKT3gamma1(Ala-Ala). *J. Clin. Invest.* **111**, 409–18 (2003).
  131. Keymeulen, B. *et al.* Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. *N. Engl. J. Med.* **352**, 2598–2608 (2005).
  132. Agace, W. W. Tissue-tropic effector T cells: generation and targeting opportunities. *Nat. Rev. Immunol.* **6**, 682–92 (2006).
  133. Hamann, A., Andrew, D. P., Jablonski-Westrich, D., Holzmann, B. & Butcher, E. C. Role of  $\alpha$ 4-integrins in lymphocyte homing to mucosal tissues in vivo. *J. Immunol.* **152**, 3282–93 (1994).
  134. Izcue, A. *et al.* Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. *Immunity* **28**, 559–70 (2008).
  135. Coombes, J. L., Robinson, N. J., Maloy, K. J., Uhlig, H. H. & Powrie, F. Regulatory T cells and intestinal homeostasis. *Immunol. Rev.* **204**, 184–194 (2005).



- 1043 136. Ogino, H. *et al.* Regulatory T cells expanded by rapamycin in vitro suppress colitis in  
1044 an experimental mouse model. *J. Gastroenterol.* **47**, 366–76 (2012).
- 1045 137. Nishio, J. *et al.* Requirement of full TCR repertoire for regulatory T cells to maintain  
1046 intestinal homeostasis. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 12770–5 (2015).
- 1047 138. Scotta, C. *et al.* Differential effects of rapamycin and retinoic acid on expansion,  
1048 stability and suppressive qualities of human CD4+CD25+FOXP3+ T regulatory cell  
1049 subpopulations. *Haematologica* **98**, 1291–1299 (2013).
- 1050 139. Doorenspleet, M. E. *et al.* Profoundly Expanded T-cell Clones in the Inflamed and  
1051 Uninflamed Intestine of Patients With Crohn's Disease. *J. Crohn's Colitis* **11**, 831–839  
1052 (2017).
- 1053 140. Gulwani-Akolkar, B. *et al.* Selective expansion of specific T cell receptors in the  
1054 inflamed colon of Crohn's disease. *J. Clin. Invest.* **98**, 1344–54 (1996).
- 1055 141. Wu, J. *et al.* Expanded TCR $\beta$  CDR3 clonotypes distinguish Crohn's disease and  
1056 ulcerative colitis patients. *Mucosal Immunol.* **11**, 1487–1495 (2018).
- 1057 142. Wu, J. *et al.* Expanded TCR $\beta$  CDR3 clonotypes distinguish Crohn's disease and  
1058 ulcerative colitis patients. *Mucosal Immunol.* **11**, 1487–1495 (2018).
- 1059 143. Harbige, J., Eichmann, M. & Peakman, M. New insights into non-conventional  
1060 epitopes as T cell targets: The missing link for breaking immune tolerance in  
1061 autoimmune disease? *J. Autoimmun.* **84**, 12–20 (2017).
- 1062 144. Rossig, C. CAR T cell immunotherapy in hematology and beyond. *Clin. Immunol.* **186**,  
1063 54–58 (2018).
- 1064 145. Voskens, C. J. *et al.* Characterization and Expansion of Autologous GMP-ready  
1065 Regulatory T Cells for TREG-based Cell Therapy in Patients with Ulcerative Colitis.  
1066 *Inflamm. Bowel Dis.* **23**, 1348–1359 (2017).
- 1067 146. Desreumaux, P. *et al.* Safety and Efficacy of Antigen-Specific Regulatory T-Cell  
1068 Therapy for Patients With Refractory Crohn's Disease. *Gastroenterology* **143**, 1207-  
1069 1217.e2 (2012).
- 1070 147. Grainge, M. J., West, J. & Card, T. R. Venous thromboembolism during active disease  
1071 and remission in inflammatory bowel disease: a cohort study. *Lancet* **375**, 657–663  
1072 (2010).
- 1073 148. Xu, Y. *et al.* In Vivo Generation of Gut-Homing Regulatory T Cells for the Suppression  
1074 of Colitis. *J. Immunol.* **202**, 3447–3457 (2019).
- 1075 149. Scalapino, K. J., Tang, Q., Bluestone, J. A., Bonyhadi, M. L. & Daikh, D. I. Suppression  
1076 of disease in New Zealand Black/New Zealand White lupus-prone mice by adoptive  
1077 transfer of ex vivo expanded regulatory T cells. *J. Immunol.* **177**, 1451–9 (2006).
- 1078 150. Scalapino, K. J. & Daikh, D. I. Suppression of Glomerulonephritis in NZB/NZW Lupus  
1079 Prone Mice by Adoptive Transfer of Ex Vivo Expanded Regulatory T Cells. *PLoS One* **4**,  
1080 e6031 (2009).
- 1081 151. He, J. *et al.* Low-dose interleukin-2 treatment selectively modulates CD4+ T cell  
1082 subsets in patients with systemic lupus erythematosus. *Nat. Med.* **22**, 991–993  
1083 (2016).
- 1084 152. Dall'Era, M. *et al.* Adoptive Regulatory T Cell Therapy in a Patient with Systemic Lupus  
1085 Erythematosus. *Arthritis Rheumatol.* (2018). doi:10.1002/art.40737
- 1086 153. Sugiyama, H. *et al.* CD4+CD25high regulatory T cells are markedly decreased in blood  
1087 of patients with pemphigus vulgaris. *Dermatology* **214**, 210–20 (2007).
- 1088 154. Taubert, R. *et al.* Intrahepatic regulatory T cells in autoimmune hepatitis are  
1089 associated with treatment response and depleted with current therapies. *J. Hepatol.*

1090 **61**, 1106–1114 (2014).

1091 155. Lee, J.-H. *et al.* The levels of CD4+CD25+ regulatory T cells in paediatric patients with  
1092 allergic rhinitis and bronchial asthma. *Clin. Exp. Immunol.* **148**, 53–63 (2007).

1093 156. Hartl, D. *et al.* Quantitative and functional impairment of pulmonary CD4+CD25hi  
1094 regulatory T cells in pediatric asthma. *J. Allergy Clin. Immunol.* **119**, 1258–1266  
1095 (2007).

1096 157. Theil, A. *et al.* T cell receptor repertoires after adoptive transfer of expanded  
1097 allogeneic regulatory T cells. *Clin. Exp. Immunol.* **187**, 316–324 (2017).

1098

1099

1100